EFFECT OF DIETARY ARACHIDONIC ACID LEVEL AND SOURCE ON NEONATAL PIGLET DEVELOPMENT

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Arachidonic acid (ARA) is a long chain polyunsaturated fatty acid that is routinely added to infant formula with docosahexaenoic acid (DHA) to support neonatal growth and development. Optimal levels of ARA in formula remain to be determined and are based largely on mean worldwide values in breast milk. The objective of this dissertation research was to determine the role of dietary ARA in neonatal development and to evaluate the optimal level in formula to support proper growth and development. Two studies with neonatal domestic pigs were carried out to achieve this objective.

The purpose of the first study was to determine the effect of the dietary ARA level on growth, clinical chemistry and immune function, and on tissue ARA and DHA accretion. On day 3 of age, formula-reared (FR) piglets were assigned to 1 of 6 milk replacer formulas containing ARA/DHA as follows (% fatty acid (FA)/FA): (a1) 0.1/1.0; (a2) 0.53/1.0; (a3-d3) 0.69/1.0; (a4) 1.1/1.0; (d2) 0.67/0.62; (d1) 0.66/0.33. No significant differences were observed among the FR groups for growth, hemogram, clinical chemistry or measures of immune status. Heart and liver ARA were responsive to dietary ARA while brain and retina were not. Heart ARA was particularly sensitive to dietary ARA. In the second study, we evaluated two ARA oils for potential use in infant formula compared with a third, commercially available ARA oil. Diets

were fed on days 3 – 22 of life and supplied ARA at 0.64% FA and DHA at 0.34% FA. We observed no toxicological effects or differences in growth, and concluded that the experimental ARA oils are safe and bioequivalent to the commercially available ARA source.

Overall, results from this dissertation research suggest that the dietary ARA level has a negligible effect on growth and development of the central nervous and immune systems when DHA is supplied near the high end of human breast milk levels (1% FA). The unique responsiveness of the heart to dietary ARA level revealed that ARA accretion is limited by dietary supply. Further investigations are warranted to determine the significance of ARA status on immediate and long term cardiac physiology.

BIOGRAPHICAL SKETCH

Cynthia Tyburczy was born on November 10, 1978 in Red Bank, New Jersey to Kathleen and Joseph Tyburczy. She grew up in Harrington Park, New Jersey and graduated from Northern Valley Regional High School in Old Tappan in 1997. As a child, she enjoyed drawing, playing softball, being a member of the Girl Scouts and cooking with her father. She developed a passion for animals early on that was fostered by frequent trips to zoos and aquariums, and horseback riding lessons.

Cynthia attended Rutgers University after high school, but soon decided to suspend her academic training to travel and attend live musical performances. She moved to the mountains of Colorado and on June 2, 2001, was blessed with the friendship of her puppy, Jackson. It was her passion for learning that led her to Cornell University where she finished her B.S. and earned her M.S. in Animal Science under the supervision of Dr. Dale Bauman studying the bioactivity and metabolism of *trans* 18:1 fatty acids. Cynthia joined the laboratory of Dr. J. Thomas Brenna where she began her doctoral work learning covalent adduct chemical ionization tandem mass spectrometry, and was matched with a project studying arachidonic acid requirements in neonatal pigs, that soon became the primary focus of her dissertation research. Currently, Cynthia has eight first author publications, and has co-authored 18 additional articles, reviews, conference proceedings & abstracts.

In her free time, Cynthia enjoys hiking with Jackson, beer, wine and cheese tasting, traveling, attending live musical performances, watching kung-fu movies, swimming and going to the beach. Post-graduation she aspires to learn to play the piano and develop a green thumb.

I dedicate this dissertation to my grandmother, Genevieve Tyburczy, whose battle with heart disease inspired me to pursue a career in lipid nutrition where my contributions could help to reduce the risk of chronic disease in future generations.

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LIST OF ABBREVIATIONS

ARA	Arachidonic acid
AST	Aspartate aminotransferase
DHA	Docosahexaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FR	Formula-reared
GRAS	Generally regarded as safe
HP	Haptoglobin
hsCRP	Highly-sensitive C-reactive protein
HUFA	Highly unsaturated fatty acid
LCPUFA	Long chain polyunsaturated fatty acid
MR	Maternal-reared
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
RAO	Refined arachidonic acid-rich oil
RBC	Red blood cell
SAA	Serum amyloid A
SFA	Saturated fatty acid

CHAPTER ONE

NEGLIGIBLE EFFECT OF DIETARY ARACHIDONIC ACID (ARA) LEVELS ON GROWTH, CLINICAL CHEMISTRY AND IMMUNE FUNCTION IN DOMESTIC PIGLETS*

Abstract

In the U.S., infant formulas with arachidonic acid (ARA) and docosahexaenoic acid (DHA) contain these long chain polyunsaturated fatty acids (LCPUFA) in a 2:1 ratio and comprising about 0.67% and 0.32% of total fatty acids (FA). Higher levels of dietary DHA appear to provide some advantages in visual or cognitive performance. This study evaluated the effect of the dietary ARA level on growth, clinical chemistry and immune function when DHA is at the high end of human breast milk levels. On day 3 of age, formula-reared (FR) piglets were matched for weight and assigned to one of six milk replacer formulas. Diets varied in the ratio of ARA/DHA as follows (% FA/FA): (a1) 0.1/1.0; (a2) 0.53/1.0; (a3-d3) 0.69/1.0; (a4) 1.1/1.0; (d2) 0.67/0.62; (d1) 0.66/0.33. A seventh group was maternal-reared (MR) and remained with the dam during the study. Blood collection and body weight measurements were performed weekly and piglets were sacrificed on day 28 of age. No significant differences were found among any of the FR groups for formula intake, growth, hemogram, clinical chemistry or immune status measurements. Mean body weight of MR pigs on day 28 of age was 2.4 kg less than the FR pigs. A few differences in clinical chemistry and immune

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status between MR and FR groups likely reflected a difference in growth rate. We conclude that the dietary ARA level has a negligible effect on growth, clinical chemistry and immune function in domestic piglets when DHA is supplied at 1.0% FA, and confirm that DHA has no effect when ARA is at 0.66%.

Introduction

Arachidonic acid (ARA, 20:4n-6) is a natural component of human breast milk and is routinely added to infant formulas along with docosahexaenoic acid (DHA, 22:6n-3). Both are long chain polyunsaturated fatty acids (LCPUFA) that contribute to normal growth and development during the perinatal period, serving as essential structural components of central nervous tissue and precursors for lipid signaling molecules (1,2). In the U.S., conventional infant formulas include ARA and DHA at target levels of 0.66% and 0.32% of total fatty acids (FA), respectively. World-wide levels in breast milk prove more variable, however, comprising 0.47 ± 0.13% (range 0.24 – 1.0%) and 0.32 ± 0.22% (range 0.06 – 1.4%) of total FA (3). The range of ARA and DHA in breast milk is likely due primarily to maternal diet. As a guide for infant formulas, the world-wide variability in breast milk levels presents a challenge for optimizing the FA composition of formula to support proper development of the infant central nervous and immune systems.

Clinical studies in human infants that are designed to determine the optimal level of LCPUFA in formula are limited in the toxicological parameters that they can measure. Here, studies have demonstrated visual and cognitive benefits of LCPUFA intake to formula-fed infants, compared with non-supplemented formula-fed infants (4). For these studies, DHA was

investigated in the range of 0.2 – 1.0% of total FA, and results provide convincing evidence for maintaining DHA in formula at levels ≥ 0.3%. Levels of DHA up to 1.0% of total FA have been reported as generally safe for infants 1-6 months of age, while preclinical studies in weanling rats report no adverse effects on growth or development with DHA intakes up to 5900 mg/kg/d (5). On the other hand, studies addressing the level of ARA or the ARA/DHA ratio in formula are rare and limited to only a few preclinical studies where ARA and DHA were fed together in a 2:1 ratio (6-8). ARA and DHA are thought to compete for tissue incorporation, including liver (9) and may influence each other's physiological effects (10,11). The effect of this competition on functional outcomes associated with ARA, particularly in the context of high dietary DHA, remains unclear.

The absence or presence of DHA and ARA in piglet formula can modulate neonatal immune responses (12), but dose-response effects have not been examined. Three immune status measures were included in this study to examine immunization response, immune maturation and inflammatory state. These measures were selected to be applicable to both piglet and infant immune function. For example, if the dietary treatments modulate immunization response in the piglets, clinical trials could then follow-up by monitoring vaccine responses in infants. For immune maturation, it is known that early life serum IgG and IgA concentrations increase as exogenous antigen exposures accumulate (13). This applies to piglets as well because gnotobiotic pigs have significantly lower serum IgA and IgG compared to conventionally-raised pigs with a typical gut microflora (14). Thus, serum IgA or IgG may provide a novel but rough comparison of net immunological maturation across dietary treatments. Finally, higher intakes of

DHA are anti-inflammatory in humans and animal models (2). It is not known if increasing the dietary ratio of ARA to DHA in piglet formula could produce a relatively pro-inflammatory state. A wide range of clinical and subclinical inflammatory events can trigger the liver to produce serum acute phase proteins. Three different acute phase proteins, highly-sensitive C-reactive protein, serum amyloid A and haptoglobin (hsCRP, SAA and Hp), were monitored because they can differ in sensitivity to detect systemic inflammation (15).

To date, there have been no ARA dose-response studies against a background of high dietary DHA in rapidly growing, suckling animals. Our objective was to determine the effect of the dietary ARA level on growth, clinical chemistry and immune function when DHA is constant and near the high end of human breast milk levels (i.e. 1.0% total FA). A dose-response for DHA was also included with normal levels of ARA. Domestic piglets were used because of their metabolic similarities to humans as well as their rates of perinatal brain growth (16). Human breast milk is considered the gold standard of nutrition for the developing infant, and because the FA composition and nutrient content of sow milk closely mimics human breast milk, an additional group of piglets were included as a maternal-reared (MR) reference treatment.

Materials and methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Cornell University. Domestic piglets were selected for gender and weight from 15 sows at the swine facility at Cornell

University. One day before the scheduled, term farrowing date, pregnant sows were injected with 2 cc of Lutalyse (Pfizer Animal Health, Kalamazoo, MI) to obtain a block of piglets with the same birth date. Piglets were processed according to standard facility practices (i.e. intramuscular injection of iron dextran (100 mg) and penicillin G (½ cc), tooth clip, tail dock, ear notch) and remained with the sow until day 3 of age, at which time they were matched for weight and assigned to one of six milk replacer diets (n=8 per diet). The formula-reared (FR) piglets were housed individually in raised metal cages and maintained on a 16/8 hr light/dark cycle. MR piglets remained with their dam at the swine facility for the duration of the study.

Diets

Milk replacer formula consisting of 60% experimental diet (Research Diets, Inc., New Brunswick, NJ) and 40% Birthright baby pig milk replacer (Ralco Nutrition, Inc., Marshall, MN) was fed to FR piglets on days 3 – 28 of age. Diets were designed to meet or exceed nutrient requirements for growing pigs 3 – 5 kg in body weight (17). The nutrient composition of the Birthright baby pig milk replacer is published elsewhere (18). A detailed ingredient composition and nutrient analysis of the experimental diets is listed in Table 1.1. The four-oil blend consisting of palmolein, soy, coconut and high oleic sunflower oils that is currently used in the commercial human infant formula Enfamil (Mead-Johnson Nutrition, Evansville, IN) made up the base oil for the experimental diets. LCPUFA were supplied by ARASCO and DHASCO single-cell oils (Martek Biosciences, Corp., Columbia, MD), derived from the marine micro-algae *C. cohnii* and the fungus *M. alpina*, respectively. As fed, the milk replacer formula had a caloric density of 0.7 kcal/ml and a

macronutrient analysis as follows (gram %): protein 26.7%, fat 21.9% and carbohydrate 38.9%. Formulas were constituted daily by adding 1.0 L water to 180 g dry diet and stored at 3-4˚C until feeding. Fresh formula was provided three times per day in stainless steel bowls fixed into the cage doors and water was offered ad libitum. FR piglets were fed at 80% ad libitum intake, based on pilot data, to moderately exceed growth rates of the MR piglets (19). Formula intakes were recorded daily.

The FA composition of the milk replacer formula and sow milk (day 14 in lactation) is presented in Table 1.2. Diets varied in the ratio of ARA/DHA as follows (% FA/FA): (a1) 0.1/1.0; (a2) 0.53/1.0; (a3-d3) 0.69/1.0; (a4) 1.1/1.0; (d2) 0.67/0.62; (d1) 0.66/0.33 (conventional infant formula), and corresponded to the following ARA/DHA intakes (mg/mg per 100 kcal of formula): (a1) 3.2/45.3; (a2) 24.0/45.3; (a3-d3) 31.3/45.3; (a4) 49.0/45.3; (d2) 30.4/28.1; (d1) 29.9/14.9. Diets a1-a4 are a dose-response for ARA against DHA constant at 1.0%, while diets d1-d3 are a dose-response for DHA against ARA constant at 0.67%. Diets d2 and a4 contain ARA and DHA at a 1:1 ratio, but at two different dietary levels, 0.67% and 1.0%, respectively. FA composition of the diets and sow milk was determined by gas chromatography. FA methyl esters (FAME) were prepared from dry diet and 50 µl sow milk by the one-step hydrolysis, extraction, and methylation procedure (20) as modified previously (21) and quantified on a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a BPX70 fused silica column (25 m x 0.22 mm i.d. x 0.25 µm film; SGE Inc., Austin, TX). A detailed protocol for the One-step method is presented in Appendix II. An equal weight FAME mixture was used to verify response factors. FAME were structurally identified by

covalent-adduct chemical ionization mass spectrometry on a Saturn 2000 mass spectrometer (Varian, Inc., Walnut Creek, CA) (22).

Sampling

Body weights measurements were taken weekly. Non-fasted blood samples were collected from the anterior vena cava into vacutainer tubes (BD, Franklin Lakes, NJ) on days 3, 7, 14 and 21 of age. Overnight-fasted blood samples were collected on day 28 of age, and an additional aliquot of blood was collected into EDTA-containing tubes for hemogram analysis. Serum was harvested by centrifuging clotted blood at 2800 rpm and 4˚C for 10 min. Piglets were sacrificed on day 28 of age via an intravenous injection of Fatal Plus (1 ml/4.54 kg body weight; Vortech Pharmaceuticals, Dearborn, MI, USA) followed by exsanguination. Organs were removed, weighed, and samples flash frozen within 10 min of cessation of heart beat.

Clinical chemistry and hemogram analysis

Serum and EDTA-blood from 28-day-old piglets were delivered to the Animal Health Diagnostic Center (College of Veterinary Medicine, Cornell University) for analysis of clinical chemistry and hemogram parameters.

Mycoplasma hyopneumoniae vaccination

Piglets were vaccinated against *M. hyopneumoniae* (RespiSure-One, Pfizer Animal Health, Kalamazoo, MI) on day 7 of age. Serum samples collected on days 21 and 28 of age (14 and 21 days post-immunization) were analyzed for antibodies to *M. hyopneumoniae*. *M. hyopneumoniae* immunity protects against a common respiratory disease in swine that is not endemic at

Table 1.1. Ingredient composition and nutrient analysis of experimental diets

Composition	Content
Dietary ingredient, % dry matter	
Dried skim milk	55.0
Oil blend	23.9
Calcium sodium caseinate	9.6
Vitamin mix[1]	4.8
Mineral mix[2]	4.8
Xanthan gum	1.4
Methionine, DL	0.5
Nutrient analysis, % kcal	
Protein	24.3
Carbohydrate	28.7
Fat	47.0

[1]The mineral mix contained (g/kg): 257.8 sucrose, 615.8 dibasic calcium phosphate, 106.7 sodium chloride, 11.8 ferric citrate, 4.0 zinc carbonate, 3.4 magnesium oxide, 0.4 cupric carbonate, 0.2 manganous carbonate, 0.1 potassium iodate, 0.01 sodium selenite.

[2]The vitamin mix contained (g/kg): 910.1 sucrose, 75.0 choline bitartrate, 6.2 vitamin E acetate (50%), 3.4 vitamin A acetate (500,000 IU/g), 1.5 vitamin D3 (100,000 IU/g), 1.3 vitamin B12 (0.1% mannitol), 0.9 pantothenic acid (d, calcium), 0.9 niacin, 0.4 biotin (1%), 0.1 riboflavin, 0.1 thiamine HCl 0.03 menadione sodium bisulfite, 0.01 folic acid, 0.01 pyridoxine HCl.

Table 1.2. FA composition of milk replacer formulas and sow milk[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR
ARA/DHA	0.1/1.0	0.53/1.0	0.69/1.0	1.1/1.0	0.67/0.62	0.66/0.33	0.74/0.01
				FA (% total FA)			
Σ SFA + MUFA	79.86	79.94	79.43	79.18	80.02	80.02	83.94 ± 3.16
ARA	0.09	0.53	0.69	1.06	0.67	0.66	0.74 ± 0.02
DHA	1.00	1.02	1.01	1.04	0.62	0.33	0.01 ± 0.01
Σ n-6	17.50	17.52	17.95	18.15	17.76	18.00	15.21 ± 3.08
Σ n-3	2.53	2.43	2.49	2.51	2.10	1.86	0.71 ± 0.04
18:2n-6	17.25	16.83	17.11	16.93	16.90	17.19	12.05 ± 2.98
18:3n-3	1.53	1.41	1.48	1.47	1.48	1.53	0.57 ± 0.06
18:2n-6/18:3n-3	11.3	12.0	11.6	11.5	11.4	11.3	21.1

[1]Milk from two sows taken on day 14 of lactation; MR piglets remained with the sow for the duration of the study.

[2]SFA, saturated FA; MUFA, monounsaturated FA.

the Cornell University swine facility or the Large Animal Research and Teaching Unit. Respisure-One is intended to induce a protective antibody titer 14-21 days after a single injection at day 7 (23).

Enzyme linked immunosorbant assays (ELISA)

Serum samples were thawed immediately prior to use. Commercial ELISA kits were used to quantify serum total IgA, IgG, and IgM, (Bethyl Laboratories, Montgomery, TX) and the acute phase proteins: hsCRP and Hp (Kamiya Biomedical Company, Seattle, WA), and SAA (Tri-Delta Development LTD, Ireland) following the manufacturer's instructions. Antibodies to *M. hyopneumoniae* were detected by semi-quantitive ELISA following the manufacturer's instructions (HerdChek™, IDEXX Laboratories, Westbrook, ME). Serum samples were tested in duplicate, with the exception of the Hp ELISA in which two different serum dilutions (1:10,000 and 1:20,000) were averaged.

Statistical analysis

Statistical analysis was carried out using the Fit Model platform of JMP (2008 SAS Institute, 8.0) to fit mixed models. Fixed effects were diet, gender, day 3 of age body weight and the full factorial of interactions. Interaction effects were considered significant at $P < 0.10$ and fixed effects at $P < 0.05$. For each parameter analyzed, effects not considered significant were removed from the final model. Random effects were litter and animal nested within litter for repeated measures of body weight. Significance of pairwise comparisons was determined using the Student's t-test. Linear regression analysis was performed to determine the relationship between clinical chemistry and

hemogram parameters and the dietary content of ARA and DHA. Values are reported as means ± SD.

Results

Clinical observations, feed intake and growth

All 56 piglets remained on the study until day 28 of age, and only one piglet (diet a3-d3) required antibiotic treatment during the study, which was due to developing an abscess on its toe. Total formula intakes averaged 51.6 ± 0.1 L and were unaffected by diet. There were small differences in body weight among the FR groups on day 7 of age with a1 piglets (0.1/1.0% ARA/DHA) weighing significantly less than all other FR groups, as shown in Figure 1.1. By day 14 of age, this difference in body weight was abolished and the a1 piglets showed body weights similar to all other FR groups for the remainder of the study. Further, there were no differences in body weight among any of the FR groups on days 14, 21 or 28 of age. The MR group was heavier than the FR groups on day 7 of age, but the MR group weighed the least at days 21 and 28 of age. On day 28 of age, mean weight for all FR piglets was 10.3 ± 0.6 kg, while MR piglets weighed 7.9 ± 0.7 kg.

Relative organ weights (weight as a percentage of final body weight) from piglets on day 28 of age are presented in Figure 1.2 (Panel A). There were no differences in relative organ weights among any of the FR groups; however, compared with the MR group, FR piglets had greater relative weights for liver. Absolute brain weights (Figure 1.2, Panel B) were similar across all diets, while relative brain weight was greatest for MR piglets compared with the FR groups.

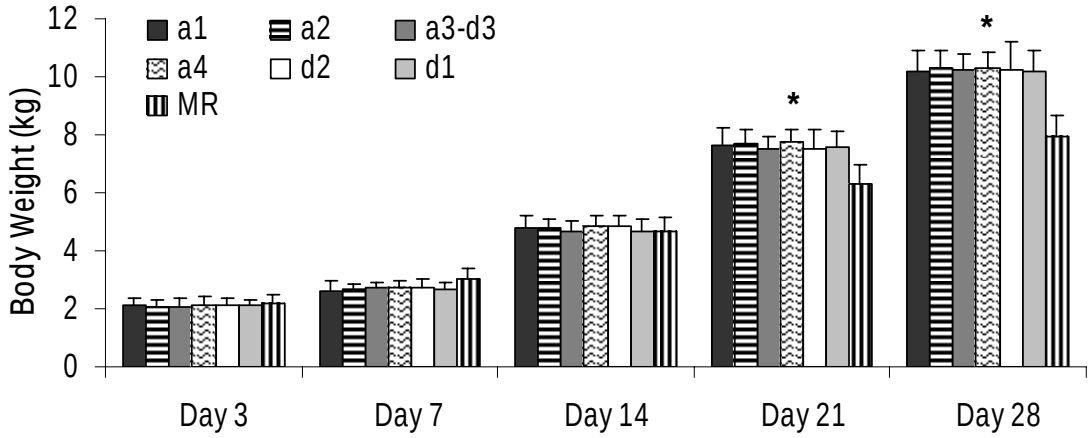

Figure 1.1. Body weight measurements by piglet day of age. Values (means ± SD) not sharing a common letter are significantly different ($P < 0.05$); * values significantly different between maternal-reared (MR) piglets and all formula-reared groups ($P < 0.05$).

Hemogram and clinical chemistry

Data for the hemogram analysis are presented in Figure 1.3. Mean values for hematocrit and red blood cell count were 35 ± 4% and 6.7 ± 0.6 mill/µl, respectively, and were unaffected by diet. Small differences were apparent among the FR groups for mean cell hemoglobin and red cell distribution width. There were no differences among the FR groups for hemoglobin, mean cell hemoglobin concentration and mean cell volume; however, FR groups significantly differed from the MR piglets for these values with the MR group presenting a more favorable hemogram status. For all piglets, platelet count averaged 1022 ± 259 thou/µl and was unaffected by treatment.

Total white blood cell count averaged 9.8 ± 3.3 thou/µl and was unaffected by diet. There were no differences among diets for segmented neutrophils, monocytes, eosinophils or basophils (data not presented).

Lymphocyte count was highest in a4 piglets; however, the range of means (4.9 – 6.8 thou/µl) across all diets was within the normal reference range for nursery piglets (24).

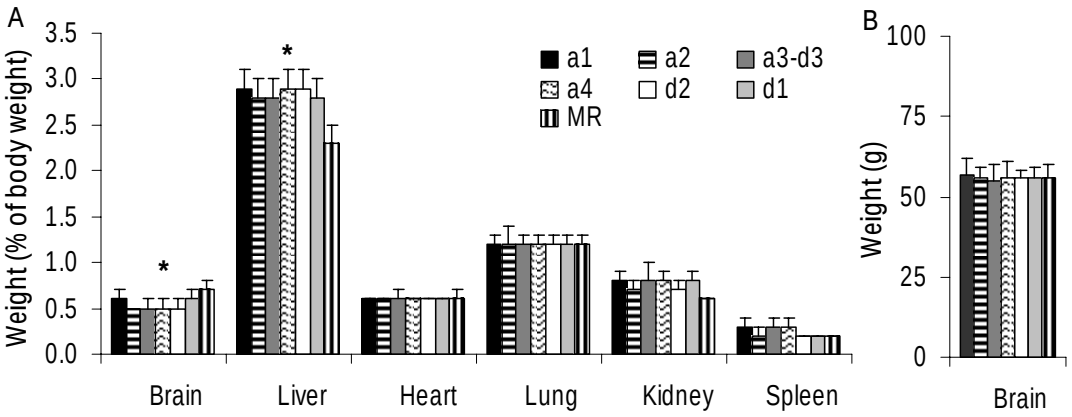

Figure 1.2. Relative organ (A) and absolute brain (B) weights from piglets on day 28 of age. *Values (means ± SD) significantly different between maternal-reared (MR) piglets and all formula-reared groups ($P < 0.05$).

Consistent with the hemogram analysis, only small differences in the clinical chemistry profile were apparent among the FR groups, including values for serum chloride, albumin and globulin, as listed in Table 1.3. While statistical differences were observed for these parameters, values fall within the normal reference ranges for nursery piglets (25,26) and thus were likely to be biologically insignificant. Also consistent with the hemogram analysis, clinical chemistry values related to serum iron status (i.e. serum iron, total iron binding capacity and percent saturation) were different between MR and FR groups and indicated a more positive serum iron status for the MR piglets. Additional serum clinical chemistry parameters that were not significantly different among any of the dietary treatments were creatinine, aspartate

aminotransferase, glutamate dehydrogenase, alkaline phosphatase, gamma-glutamyl transferase, sorbitol dehydrogenase and creatinine kinase.

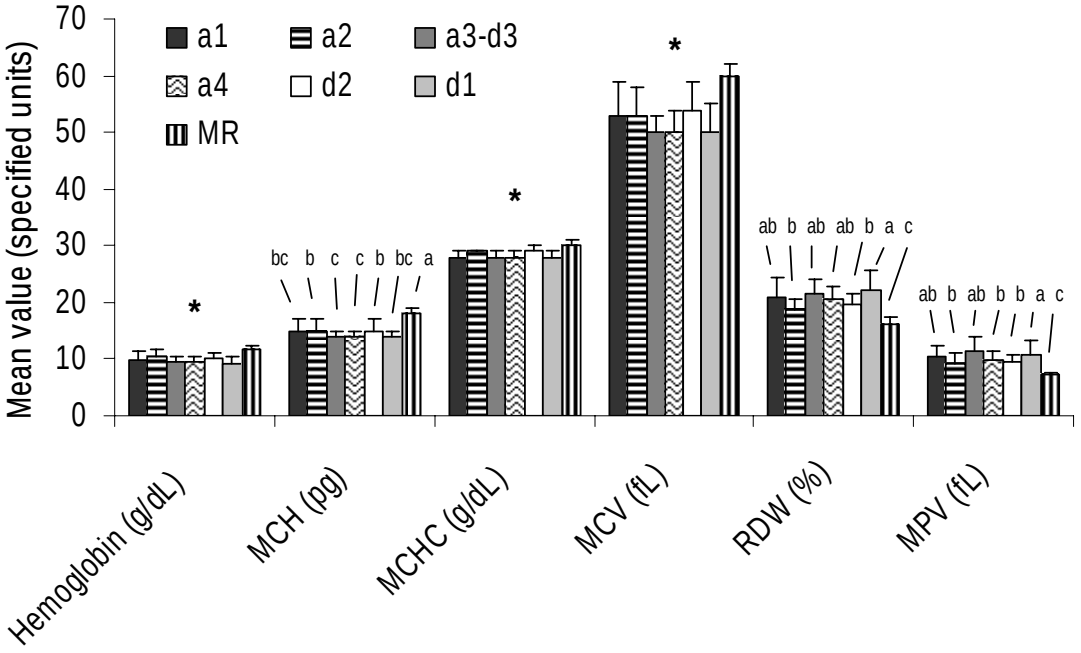

Figure 1.3. Profile of hemogram parameters from piglets on day 28 of age. Values (means ± SD) not sharing a common letter are significantly different ($P < 0.05$); * values significantly different between maternal-reared (MR) piglets and all formula-reared groups ($P < 0.05$). MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; MPV, mean platelet volume; MR, maternal-reared; RDW, red cell distribution width.

Immunization response & measurements of immune status

Table 1.4 presents a summary of immune status measurements. All piglets were negative for antibodies to *M. hyopneumoniae* on day 28 of age and there were no significant differences in titers to *M. hyopneumoniae* by dietary treatment. Serum IgA levels in the MR piglets were significantly higher on day 28 of age than all FR groups ($P < 0.001$), but IgA levels did not differ among any of the FR groups. Serum IgM and IgG levels on day 28 of age were unaffected by diet. There were minor differences in SAA levels on day 7

Table 1.3. Summary of clinical chemistry profiles from piglets on day 28 of age[1,2]

Diet	a1	a2	a3-d3	a4	d1	d2	MR	
ARA/DHA (%, FA/FA)	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
Glucose (mg/dL)	158 ± 30	152 ± 32	159 ± 27	156 ± 33	162 ± 27	169 ± 32	131 ± 8	NS
BUN (mg/dL)	6 ± 1	6 ± 2	6 ± 2	6 ± 2	6 ± 1	6 ± 2	8 ± 1	NS
Creatinine (mg/dL)	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	NS
Total protein (g/dL)	5.2 ± 0.3	5.0 ± 0.3	5.0 ± 0.3	5.1 ± 0.4	5.0 ± 0.3	5.3 ± 0.3	4.7 ± 0.3	NS
Total bilirubin (mg/dL)	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	NS
Na (mEq/L)	143 ± 4	143 ± 3	144 ± 3	145 ± 3	143 ± 2	144 ± 2	141 ± 2	NS
K (mEq/L)	5.9 ± 0.7	6.2 ± 0.7	6.2 ± 0.8	6.2 ± 0.8	5.9 ± 0.8	5.9 ± 0.5	5.2 ± 0.9	NS
Cl (mEq/L)	100 ± 2^{c}	101 ± 1^{bc}	102 ± 2^{ab}	101 ± 2^{ab}	100 ± 2^{bc}	100 ± 2^{c}	105 ± 2^{a}	0.02
Bicarbonate (mEq/L)	19 ± 5	21 ± 5	20 ± 4	20 ± 6	21 ± 4	19 ± 5	23 ± 2	NS
Ca (mg/dL)	11.3 ± 0.7	11.3 ± 0.6	11.1 ± 0.5	11.2 ± 0.7	11.1 ± 0.4	11.3 ± 0.3	10.6 ± 0.2	NS
P (mg/dL)	10.8 ± 1.0^{a}	11.0 ± 0.9^{a}	11.1 ± 0.8^{a}	10.9 ± 0.8^{a}	11.3 ± 1.1^{a}	10.9 ± 0.7^{a}	8.1 ± 0.5^{b}	<.0001
Mg (mEq/L)	1.9 ± 0.3	1.9 ± 0.3	1.9 ± 0.2	1.9 ± 0.3	2.0 ± 0.4	2.0 ± 0.2	1.9 ± 0.2	NS
Fe (µg/dL)	22 ± 8^{b}	25 ± 9^{b}	18 ± 2^{b}	24 ± 6^{b}	23 ± 4^{b}	41 ± 50^{b}	90 ± 40^{a}	<.0001
TIBC (µg/dL)	814 ± 57^{a}	764 ± 67^{a}	818 ± 86^{a}	803 ± 77^{a}	771 ± 59^{a}	800 ± 100^{a}	397 ± 50^{b}	<.0001
Saturation (%)	3 ± 1^{b}	4 ± 1^{b}	2 ± 0^{b}	3 ± 1^{b}	3 ± 1^{b}	6 ± 7^{b}	23 ± 10^{a}	<.0001
Albumin (g/dL)	4.1 ± 0.2^{ab}	4.1 ± 0.2^{ab}	4.0 ± 0.3^{b}	4.1 ± 0.3^{ab}	4.0 ± 0.2^{ab}	4.3 ± 0.2^{a}	3.4 ± 0.3^{c}	0.0002
Globulin (g/dL)	1.1 ± 0.3^{a}	1.0 ± 0.2^{c}	1.0 ± 0.2^{abc}	1.0 ± 0.2^{abc}	1.0 ± 0.2^{bc}	1.0 ± 0.2^{a}	1.3 ± 0.4^{abc}	0.02
Albumin:Globulin	4.1 ± 1.2	4.4 ± 1.0	4.2 ± 1.1	4.2 ± 0.6	4.4 ± 0.9	4.3 ± 0.8	2.9 ± 1.0	NS

[1]Values (means ± SD) not sharing common superscript are significantly different ($P < 0.05$); NS, not significant ($P > 0.05$).

[2]Reference ranges for nursery piglets have been previously reported (25,26).

[3]BUN, blood urea nitrogen; TIBC, total iron binding capacity.

Table 1.4. Summary of immune status measurements in piglets fed varying levels of ARA and DHA[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR	*P*
ARA/DHA (% FA/FA)	0.1/1.0	0.53/1.0	0.69/1.0	1.06/1.0	0.67/0.62	0.66/0.33	0.74/0.01	
M. hyopenmoniae (sample/positive)	0.1 ± 0.1	0 ± 0.1	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.3	NS
IgG (µg/ml)	4866 ± 935	4439 ± 967	4293 ± 1018	4952 ± 933	4158 ± 781	4865 ± 942	5903 ± 1031	NS
IgM (µg/ml)	540 ± 254	470 ± 186	551 ± 257	525 ± 161	534 ± 216	481 ± 176	768 ± 186	NS
IgA (µg/ml)	160 ± 71[b]	167 ± 95[b]	168 ± 63[b]	157 ± 70[b]	164 ± 69[b]	132 ± 56[b]	408 ± 99[a]	0.0008
hsCRP (µg/ml)								
Day 3	16.4 ± 10	17 ± 10.3	20.1 ± 10.6	12.5 ± 6.2	10.6 ± 7	11.1 ± 5.9	17.7 ± 9.4	NS
Day 7	10.5 ± 2.5	10 ± 3.4	11 ± 6	11.3 ± 3.4	8.8 ± 3.4	8.9 ± 1.6	10.6 ± 2.7	NS
Day 14	9 ± 3.3	11.3 ± 4.8	10.4 ± 3.5	9 ± 3.7	6.6 ± 2.8	10.6 ± 1.8	12.5 ± 2.5	NS
Day 21	14.4 ± 6.8	16.2 ± 11.9	12.7 ± 4.5	13.1 ± 3	10.2 ± 5.1	14.8 ± 4.3	13 ± 2.6	NS
Day 28	13.5 ± 6	13 ± 4.6	14.6 ± 6.2	12.4 ± 3.4	10.6 ± 5.3	15.8 ± 6.8	15.2 ± 4.6	NS
SAA (µg/ml)								
Day 3	23.4 ± 64.4	19.5 ± 29.2	38.6 ± 56.4	26.1 ± 47.9	5.3 ± 14.4	1.6 ± 2.5	17.7 ± 49.3	NS
Day 7	17 ± 29.0[a]	9.5 ± 7.3[b]	21.1 ± 20.4[ab]	15.5 ± 23.0[b]	6.8 ± 4.6[b]	10.2 ± 9.9[b]	6.4 ± 7.1[b]	0.03
Day 28	2.5 ± 3.8	2.8 ± 3.8	5.7 ± 5.1	1.9 ± 1	2 ± 1.9	3 ± 4	0.9 ± 0.4	NS
Hp(µg/ml)	807 ± 818[a]	235 ± 176[b]	1137 ± 874[ab]	355 ± 516[b]	593 ± 1033[ab]	812 ± 706[a]	n/a[2]	0.02

[1]Hp, haptoglobin; hsCRP, highly-sensitive C-reactive protein; SAA, serum amyloid A.

[2]Values below limit of detection.

of age, with the a1 (low ARA) group having significantly higher levels than most other groups. SAA levels, however, were unaffected by diet on day 3 and 28 of age. Likewise, there were minor, statistically significant differences in serum Hp levels among FR groups on day 28, although no discernable pattern among diets was apparent. Hp in the MR piglets was below detectable limits. Serum hsCRP levels were similar among all groups on days 3, 7, 14, 21 and 28 of age.

Discussion

Human breast milk is considered the gold standard of nutrition for the developing infant. There is broad consensus that human milk composition serves as a model for infant formula. All breast milks naturally contain ARA and DHA but the range of reported concentrations is wide and thus studies of optimal levels within the natural range are relevant to setting composition guidelines. With interest in optimizing the formula content of ARA and DHA for proper development of the infant central nervous and immune systems, it is critical to determine the interaction between these two LCPUFA and their resulting effect on growth and development. The present study was carried out to determine if there were any safety concerns associated with the dietary ARA level on growth, clinical chemistry and immune function in domestic piglets when DHA is constant and near the high end of human breast milk levels.

Human term and preterm infants are capable of synthesizing ARA and DHA from the dietary precursors linoleic acid (18:2n-6) and linolenic acid (18:3n-3), respectively, although rates of endogenous biosynthesis and tissue accretion are insufficient to support optimal neural and visual development in

western countries in which studies have been conducted (27). Formulas supplemented with single-cell triglyceride oils serve as highly bioavailable sources of LCPUFA and enhance plasma and red blood cell content of ARA and DHA compared with infants consuming LCPUFA-free formula, and within the range of breast-fed infants (28). However, plasma and red blood cell ARA levels serve as surrogate biomarkers for tissue status, especially with regard to neural tissue (29). Functional outcomes, including those associated with safety, are thus necessary to develop a complete picture of their effects.

Growth is considered one of the most useful clinical markers of infant development, and over the last two decades, growth outcomes have been intimately linked as a basis for addition of ARA to infant formula. Early studies showed a correlation between blood ARA and growth in preterm infants fed formula (30), and prompted investigation of the hypothesis that plasma ARA levels are crucial to growth outcomes (31). Recent studies, however, indicate that LCPUFA supplementation has a negligible effect on growth (32,33). In the present study, piglets fed 0.1% ARA had marginally lower, but statistically significant different body weights on day 7 of age compared with all other FR and MR piglets. These differences in body weight were abolished by day 14 of age and the 0.1% ARA piglets grew as well as all other FR pigs for the remainder of the study. For all piglets, the rapid rates of growth and concomitant demand for nutrients may have exacerbated the requirement for ARA during the first two weeks of life. The approximately eight-fold greater growth rate of piglets compared to human infants would be expected to amplify effects of ARA on growth, if any (34). Thus, results from the present study are consistent with recent reports in human infants and suggest a negligible effect of ARA level on growth.

M. hyopneumoniae titers did not differ among any of the dietary treatment groups. Titers were also relatively low compared to the manufacturer's data on RespiSure-One (23). The HerdChek™ ELISA kit is intended to monitor pig herds for outbreaks of *M. hyopneumoniae* and focuses on dividing sample titers into positive versus negative cases relative to a single positive control sample, and may have limited ability to detect subtle changes. Future studies using this system may benefit from different immunizations or immunization schedules to induce higher serum titers and a detection test with a broader dose-response curve.

MR piglets had significantly higher levels of serum IgA than the other dietary treatment groups. One possible explanation is that some or all of the IgA is maternally derived. However, by 24-36 hours after birth, piglets no longer transport milk immunoglobulins from the gut into circulation (35). And, based on ^{125}I labeled immunoglobulin clearance rates (36), the average half-life of maternally-derived IgA is 2.7 days. Thus, the elevated IgA in the serum of MR controls is unlikely to be of maternal origin and may reflect differences in the rate of maturation. Both serum IgG and IgM showed groupwise comparison trends similar to IgA but failed to reach significance. The lack of significant differences among the FR groups indicates altering the dietary amounts or ratios of ARA to DHA has no adverse effect on immunological maturation. Serum IgG levels, in the present study, were marginally lower than previously reported values for sow-fed piglets (37,38) but consistent with other formula-fed piglets (37). Serum IgM levels were comparable to previous studies (37,38).

Serum acute phase protein levels showed few significant differences among dietary treatment groups. Values were highly variable, especially for

Hp and SAA, had inconsistent patterns of between-group differences and, in some cases, tended to be either higher or lower than previously published data (39,40). The lack of detectable Hp and low concentrations of SAA in the MR pigs are inconsistent with previous studies of early life acute phase protein concentrations in piglets. For example, in (39), , SAA levels were slightly higher than values for our MR pigs, but showed a similar trend of decreasing levels with age. Where significant differences did exist among dietary treatment groups, e.g. day 28 Hp and day 7 SAA, they were not associated with higher concentrations of ARA relative to DHA. Thus, with a battery of three acute phase protein tests, we could not find evidence that increasing ARA relative to DHA produces a detectable pro-inflammatory state in the piglets.

We have previously examined the safety of ARA and DHA for use in formula at 0, 1, 3 and 5 times the levels currently used in conventional infant formulas (7). For the 5x diet, levels of ARA and DHA corresponded to 3.15 and 1.66% of total FA, respectively, and across the full range of ARA and DHA intakes tested, no toxicological effects were observed for growth, clinical chemistry or liver histopathology. Others have observed a similar negligible effect on growth outcomes when ARA and DHA were fed 3x the level in conventional formulas (8). Safety concerns associated with providing ARA and DHA at different ratios, particularly with regard to growth and immune status, led us to conduct the present study. Here, we observed negligible differences among all the FR groups, indicating that the dietary ARA level, when DHA is constant at 1.0% of total FA, does not influence functional outcomes associated with ARA during the neonatal period. One possible explanation for a lack of difference is that fact that the dietary content of

linoleic acid (18:2n-6), roughly 17% of total FA and at a ratio of 11.5:1 with linolenic acid (18:3n-3), supported the endogenous biosynthesis of ARA at levels sufficient for normal growth and immune function.

A superficially striking observation from the present study was the large difference in growth, hemogram and clinical chemistry parameters between the MR and FR piglets. These results were not unexpected, however, as the diet and rearing environment differed tremendously between the two groups. In terms of growth, we opted to set intakes for the FR piglets at 80% ad libitum intake based on reports of growth patterns for MR piglets (19) and data from a pilot study. This would enable a moderately faster rate of growth for the FR piglets, which was observed with a 2 kg difference in body weight between FR and MR piglets on day 28 of age.

Remarkably, absolute brain weights were unaffected by diet. However, relative brain weights expressed as brain weight as a percentage of body weight were greatest for the MR piglets, and driven entirely by the larger carcasses of the FR groups. Here, excess nutrients beyond those required for brain growth were partitioned to the FR carcass, highlighting the brain's protected growth during the perinatal period. Differences between MR piglets and the FR groups were also observed for biomarkers of blood iron status, including hemoglobin, serum iron, total iron binding capacity and percent saturation. Furugouri (41) demonstrated that rapidly growing piglets exhibit high levels of erythropoesis and have a heightened daily requirement for available iron. In the present study, the faster rates of growth observed for the FR piglets, compared with the MR group, corresponded to an elevated demand for iron that was apparently unmet and manifested as poorer blood iron status.

Current FDA GRAS Notices indicate that ARA be included in infant formula at levels at least equal to DHA (42,43), confirmed recently by recommendations of an expert panel (44). Panel recommendations also suggest that the formula content of DHA not exceed 0.5% of total FA due to a lack of evidence showing further benefit beyond the 0.3% level. Data from the present study show no toxicological effects of DHA when supplemented up to 1% of total FA, and extend the current body of literature to show that comparable levels of ARA are not required to match high DHA to support normal growth and immune function. In Chapter Two, we examine the influence of dietary ARA level on tissue ARA and DHA accretion. It would be of further interest to determine the competition between these LCPUFA at the tissue level and to determine if dietary ARA level influences functional outcomes associated with DHA, such as visual acuity or cognition.

In conclusion, milk replacer formulas supplemented with varying levels of ARA and DHA equally supported normal growth, development and immune function of rapidly growing domestic piglets up to 28 days of age. Dietary ARA level, comprising 0.1 – 1.0% of total FA, and fed concomitantly with 1.0% DHA produced no toxicological effects on any hemogram, clinical chemistry or immune function parameters. The lack of adverse or toxicological effects in piglets consuming the low ARA diet (0.10% total FA) may be due to adequate endogenous biosynthesis of ARA from 18:2n-6.

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CHAPTER TWO

HEART ARACHIDONIC ACID (ARA) IS UNIQUELY SENSITIVE TO DIETARY ARA AND DOCOSAHEXAENOIC ACID (DHA) CONTENT IN DOMESTIC PIGLETS*

Abstract

Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are commonly added to infant formula to support neonatal growth and development. Higher levels of these long chain polyunsaturated fatty acids (FA) in formula may provide some advantage in visual or cognitive performance. This study determined the sensitivity of heart and brain ARA to the dietary ARA level in a dose-response design with constant, high DHA. On d3 of age, formula-reared (FR) pigs were assigned to 1 of 6 milk replacer formulas that varied in ARA/DHA as follows (%, FA/FA): (a1) 0.1/1.0; (a2) 0.53/1.0; (a3-d3) 0.69/1.0; (a4) 1.1/1.0; (d2) 0.67/0.62; (d1) 0.66/0.33. A seventh group was maternal-reared. On d28 of age, piglets were sacrificed and organs harvested. Higher levels of dietary ARA were associated with increased heart and liver ARA, while brain ARA remained unaffected. Dietary ARA had no effect on tissue DHA accretion. Higher levels of dietary DHA resulted in higher DHA in the heart, liver and neural tissue, as well as reduced liver ARA. The heart was particularly sensitive, with pigs in the intermediate groups having different ARA values (a2, 18.6 ± 0.7%; a3, 19.4 ± 1.0%) and a 0.17% increase in dietary ARA resulted in a 0.84% increase in heart ARA. We

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conclude that the heart has a unique responsiveness to the dietary ARA level. Further investigations are warranted to determine the clinical significance of heart ARA level in the developing neonate.

Introduction

Arachidonic acid (20:4n-6; ARA) and docosahexaenoic acid (22:6n-3; DHA) are two long chain polyunsaturated fatty acids (LCPUFA) that contribute to normal growth and development during the perinatal period. Both are natural components of breast milk and since 2002, these LCPUFA have been added to infant formulas in the U.S. at target levels of 0.64% and 0.32% of total fatty acids (FA) (1). Mean worldwide levels of ARA and DHA in breast milk average 0.47 ± 0.13% total FA (range: 0.24 – 1.0% FA) and 0.32 ± 0.22% FA (range: 0.06 – 1.4% FA), respectively, with individual variability in DHA attributed largely to maternal diet (2). Inevitably, the wide variation in breast milk LCPUFA as well as the relative inaccessibility to human infant tissue presents a major challenge for optimizing levels of ARA and DHA in formula to support neonatal development.

It is well-established that the addition of ARA and DHA to formula enhances blood LCPUFA levels equivalent to those of breastfed infants and improves visual acuity and cognitive performance compared with infants fed LCPUFA-free formula (3-6). Functional outcome studies with infants provide clear evidence for the addition of DHA at 0.32% FA (7,8), while higher DHA levels may offer further benefit for neural development, especially in target populations (9-11). Optimal levels of ARA remain to be determined at any DHA intake and are based largely on mean global levels in breast milk (12). ARA comprises approximately 10 – 12% total FA in human infant central

nervous tissue (cerebral cortex and retina) appears to be influenced to a greater extent by postnatal age than dietary ARA supply (13). Animal models suggest a relative resistance of brain and retina ARA to dietary ARA intake, possibly as a mechanism for regulating its potent bioactivity (14,15). ARA levels in the heart, liver and blood-borne pools vary with ARA intake and may compete with n-3 LCPUFA for tissue incorporation (3,16-23), especially in the liver (20,23).

The sensitivity of heart ARA to dietary ARA content has been reported a few times but has not been systematically studied (17-19,21). In pigs (21), dietary ARA and DHA were maintained at a constant 2:1 ratio and total levels varied up to 5 times the target levels in formula. While liver ARA increased with only the highest ARA intakes, heart ARA responded in a direct dose-response manner to each incremental increase in dietary ARA. Further, heart ARA more than doubled with the highest ARA level compared with pigs fed LCPUFA-free formula.

We tested the hypothesis that heart and brain ARA is sensitive to dietary ARA in a dose-response design with DHA constant at a high physiological level. We examined four levels of ARA ranging from 0.09 to 1.0% total FA against a background of 1.0% DHA. Intermediary levels were 0.53 and 0.67% total FA, where 0.53% is slightly above the worldwide ARA mean in human breastmilk and 0.67% is among the highest values reported. A DHA dose-response was also included to determine the influence of dietary DHA level on tissue ARA incorporation when ARA levels are comparable to those added in conventional formula (0.65% ARA). A randomized maternal-reared (MR) reference group was included for comparison.

Materials and methods

Animals and diets

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Cornell University. On day 3 of life, domestic piglets were matched for weight and gender and assigned to one of six milk replacer diets (n = 8 per diet). Piglets in the formula-reared (FR) groups were then transferred to the Large Animal Research and Teaching Unit, where they were housed individually in raised metal cages and maintained on a 16/8 hr light/dark cycle. The seventh group was MR and remained with the dam at the swine facility for the duration of the study. Intake and growth performance is reported in Chapter One.

Milk replacer formula consisting of 60% experimental diet (Research Diets, Inc., New Brunswick, NJ) and 40% Birthright baby pig milk replacer (Ralco Nutrition, Inc., Marshall, MN) was fed to FR piglets on days 3 – 28 of age. The ingredient composition and nutrient analysis of the base experimental diet is presented in Table 2.1. A detailed nutrient composition and analysis of the Birthright baby pig milk replacer has been reported previously (24). As fed, the milk replacer formula had a caloric density of 0.7 kcal/mL and a nutrient composition as follows (% wt/wt): protein 26.7, fat 21.9 and carbohydrate 38.9. The FA composition of the milk replacer formulas and sow milk (day 14 in lactation) is presented in Table 2.2. Diets varied in the ratio of ARA/DHA as follows (%, FA/FA): (a1) 0.1/1.0; (a2) 0.53/1.0; (a3-d3) 0.69/1.0; (a4) 1.1/1.0; (d2) 0.67/0.62; (d1) 0.66/0.33 (conventional infant formula). Diets a1 – a4 are a dose-response for ARA against DHA constant at 1.0%, while Diets d1 – d3 are a dose-response for DHA against ARA constant at 0.67%.

Sampling

On day 28 of age, piglets were sacrificed via an intravenous injection of Fatal Plus (1 mL/4.54 kg body weight; Vortech Pharmaceuticals, Dearborn, MI, USA) followed by exsanguination. Organs were immediately removed, weighed and samples flash frozen in liquid nitrogen within 10 min of cessation of heart beat. Cerebral cortex and cerebellum samples were harvested from the left hemisphere of each brain prior to submerging individual hemispheres in liquid nitrogen. Internal brain regions were sampled from partially thawed left hemispheres at a later period. Tissues were stored at -80°C until FA analysis.

Lipid analysis

Fatty acid methyl esters (FAME) were prepared from ~50 mg of tissue or dry diet and 50 µL sow milk according to the One-step hydrolysis, extraction and methylation procedure (25) with previous modifications (26). A detailed protocol for the One-step procedure is presented in Appendix II. Retinas, which were suspended in physiological saline at necropsy, were first dried using a Savant SpeedVac Concentrator (Thermo Fisher Scientific, Waltham, MA). FAME were quantified on a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a BPX70 fused silica column (25 m x 0.22 mm i.d. x 0.25 µm film; SGE Incorportated, Austin, TX). FAME were structurally identified by covalent-adduct chemical ionization mass spectrometry on a Saturn 2000 mass spectrometer (Varian, Inc., Walnut Creek, CA) (27). An equal weight FAME mixture was used daily to verify response factors.

Table 2.1 Nutrient composition and analysis of experimental diets

Composition	Content
Dietary ingredient, % dry matter	
Dried skim milk	55.0
Oil blend	23.9
Calcium sodium caseinate	9.6
Vitamin mix[1]	4.8
Mineral mix[2]	4.8
Xanthan gum	1.4
Methionine, DL	0.5
Nutrient analysis, % kcal	
Protein	24.3
Carbohydrate	28.7
Fat	47.0
Caloric density (kcal/g)	4.67

[1]The mineral mix contained (g/kg): 257.8 sucrose, 615.8 dibasic calcium phosphate, 106.7 sodium chloride, 11.8 ferric citrate, 4.0 zinc carbonate, 3.4 magnesium oxide, 0.4 cupric carbonate, 0.2 manganous carbonate, 0.1 potassium iodate, 0.01 sodium selenite.

[2]The vitamin mix contained (g/kg): 910.1 sucrose, 75.0 choline bitartrate, 6.2 vitamin E acetate (50 g/100 g), 3.4 vitamin A acetate (500,000 IU/g), 1.5 vitamin D3 (100,000 IU/g), 1.3 vitamin B12 (0.1g/100 g mannitol), 0.9 pantothenic acid (d, calcium), 0.9 niacin, 0.4 biotin (1 g/100 g), 0.1 riboflavin, 0.1 thiamine HCl 0.03 menadione sodium bisulfite, 0.01 folic acid, 0.01 pyridoxine HCl.

Table 2.2. FA composition of milk replacer formulas and sow milk[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR
ARA/DHA	0.1/1.0	0.53/1.0	0.69/1.0	1.1/1.0	0.67/0.62	0.66/0.33	0.74/0.01
				FA (% total FA)			
Σ SFA + MUFA	79.86	79.94	79.43	79.18	80.02	80.02	83.94 ± 3.16
ARA	0.09	0.53	0.69	1.06	0.67	0.66	0.74 ± 0.02
DHA	1.00	1.02	1.01	1.04	0.62	0.33	0.01 ± 0.01
Σ n-6	17.50	17.52	17.95	18.15	17.76	18.00	15.21 ± 3.08
Σ n-3	2.53	2.43	2.49	2.51	2.10	1.86	0.71 ± 0.04
18:2n-6	17.25	16.83	17.11	16.93	16.90	17.19	12.05 ± 2.98
18:3n-3	1.53	1.41	1.48	1.47	1.48	1.53	0.57 ± 0.06
18:2n-6/18:3n-3	11.3	12.0	11.6	11.5	11.4	11.3	21.1

[1]Milk from two sows taken on day 14 of lactation; MR piglets remained with the sow for the duration of the study.

[2]SFA, saturated FA; MUFA, monounsaturated FA.

Statistical analysis

Values are reported as means ± SD. Statistical analysis was carried out using the Fit Model platform of JMP (2008 SAS Institute, 8.0) to fit mixed models and tested the hypothesis that all mean treatment values are not equal. Fixed effects were diet, gender, day 3 of age body weight and the full factorial of interactions. The random effect of litter was included the model. Interaction effects were considered significant at $P < 0.10$ and fixed effects at $P < 0.05$. For each parameter analyzed, effects not considered significant were removed from the final model. Significance of pairwise comparisons was determined using the Student's t-test. Linear regression analysis was performed to determine the dose-response of tissue LCPUFA to the dietary ARA and DHA level.

Results

LCPUFA accretion in liver and heart

Table 2.3 presents the FA composition of heart and liver. A detailed composition of these tissues is presented in Appendix I (Tables S1 and S2). Heart ARA increased with increasing dietary ARA. Heart ARA levels were higher in the a3 group compared with Diet a2 ($P = 0.04$) such that a 0.17% increase in dietary ARA corresponded to an increase of 0.84% in heart ARA between the two treatments. Mean heart ARA was 40% higher in the a4 group compared with a2 pigs. Liver ARA increased with increasing dietary ARA, although the greatest increase in liver ARA was observed between Diets a1 and a2.

DHA accretion in the liver reflected dietary intake with a1 – a4 pigs having the highest liver levels, d1 and d2 pigs having intermediary levels and

MR pigs having the lowest liver DHA (Table 2.3). Similarly, DHA levels in the heart reflected dietary DHA content, with a1 – a4 pigs having the highest, d1 and d2 pigs having intermediary and MR pigs having the lowest heart DHA levels ($P < 0.0001$), although for Diets a1 – a4, a2 pigs had higher heart DHA levels than the a1 and a3 groups ($P < 0.05$). MR pigs had a strikingly low DHA content (0.75 ± 0.08%) that was 6-fold lower than DHA levels in the a2 group.

In both the heart and liver, the sum of 22-carbon HUFA reflected tissue DHA content and was lowest in d1 and MR pigs ($P < 0.0001$; Table 2.3). Compared with the d3 group, d1 pigs had lower liver DHA and higher liver levels of 22:4n-6, 22:5n-6 and 22:5n-3 ($P < 0.001$). In the heart, however, d1 pigs showed increases in 22:4n-6 and 22:5n-3 compared with d3 pigs ($P < 0.0001$), while trace levels of 22:5n-6 were detected in all samples.

LCPUFA accretion in neural tissues

Tables 2.4 and 2.5 present the FA composition of neural tissue from pigs fed varying levels of ARA and DHA from day 3 – 28 of age. The detailed FA composition of each neural tissue is presented in Appendix I (Tables S3 – S8). ARA levels in the cerebral cortex, hippocampus, globus pallidus and inferior colliculus were similar across all dietary treatments. ARA levels in the cerebellum were significantly influenced by diet ($P = 0.007$) but showed no distinct pattern associated with ARA or DHA intake. A1 pigs had the lowest retina ARA levels of the FR groups, although differences among the means only reached significance for the a4, d1 and d2 groups ($P < 0.05$).

Table 2.3. FA composition of heart and liver from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	*P*
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	
Liver				*FA (% total FA)*				
ARA	13.92 ± 1.49^{d}	16.14 ± 0.87^{c}	16.12 ± 1.10^{bc}	16.88 ± 1.50^{b}	17.36 ± 0.63^{b}	17.87 ± 1.46^{a}	17.00 ± 1.36^{abc}	<.0001
22:4n-6	1.08 ± 0.29^{d}	1.15 ± 0.22^{cd}	1.27 ± 0.22^{c}	1.36 ± 0.22^{bc}	1.28 ± 0.20^{bc}	1.50 ± 0.10^{b}	2.27 ± 0.35^{a}	<.0001
22:5n-6	0.03 ± 0.06^{d}	0.06 ± 0.05^{d}	0.04 ± 0.04^{d}	0.06 ± 0.07^{d}	0.20 ± 0.09^{c}	0.37 ± 0.11^{b}	1.89 ± 0.53^{a}	<.0001
20:5n-3	0.61 ± 0.14^{a}	0.42 ± 0.15^{bc}	0.45 ± 0.10^{b}	0.36 ± 0.11^{cd}	0.34 ± 0.05^{cd}	0.31 ± 0.04^{d}	0.18 ± 0.06^{e}	<.0001
22:5n-3	0.94 ± 0.11^{c}	0.91 ± 0.09^{c}	0.94 ± 0.12^{c}	0.94 ± 0.14^{c}	1.01 ± 0.07^{c}	1.11 ± 0.16^{b}	1.35 ± 0.20^{a}	<.0001
DHA	10.77 ± 1.77^{a}	11.43 ± 1.22^{a}	10.49 ± 1.41^{a}	10.45 ± 1.79^{a}	9.65 ± 1.01^{b}	7.52 ± 0.69^{c}	2.37 ± 0.30^{d}	<.0001
∑ 22C HUFA[4]	12.82 ± 1.86^{a}	13.55 ± 1.26^{a}	12.74 ± 1.44^{ab}	12.81 ± 1.90^{ab}	12.14 ± 1.21^{b}	10.51 ± 0.81^{c}	7.88 ± 1.22^{d}	<.0001
Heart								
ARA	14.76 ± 1.07^{d}	18.57 ± 0.67^{c}	19.41 ± 1.00^{b}	20.90 ± 0.93^{a}	19.98 ± 1.38^{ab}	19.09 ± 1.88^{bc}	20.26 ± 1.20^{ab}	<.0001
22:4n-6	0.63 ± 0.09^{e}	0.71 ± 0.09^{e}	0.71 ± 0.06^{de}	0.82 ± 0.09^{cd}	0.88 ± 0.13^{bc}	0.96 ± 0.12^{b}	1.33 ± 0.13^{a}	<.0001
22:5n-6	0.00 ± 0.01^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.02 ± 0.03^{b}	0.08 ± 0.01^{a}	<.0001
20:5n-3	0.75 ± 0.09^{a}	0.54 ± 0.09^{b}	0.49 ± 0.04^{b}	0.41 ± 0.04^{c}	0.42 ± 0.04^{c}	0.32 ± 0.03^{d}	0.28 ± 0.06^{d}	<.0001
22:5n-3	0.59 ± 0.07^{d}	0.59 ± 0.08^{d}	0.57 ± 0.04^{d}	0.57 ± 0.04^{d}	0.71 ± 0.07^{c}	0.83 ± 0.13^{b}	1.24 ± 0.07^{a}	<.0001
DHA	4.81 ± 0.24^{b}	5.20 ± 0.49^{a}	4.78 ± 0.43^{b}	5.01 ± 0.25^{ab}	4.20 ± 0.64^{c}	3.00 ± 0.50^{d}	0.75 ± 0.08^{e}	<.0001
∑ 22C HUFA	6.04 ± 0.25^{bc}	6.50 ± 0.51^{a}	6.06 ± 0.4^{abc}	6.40 ± 0.23^{ab}	5.78 ± 0.72^{c}	4.81 ± 0.59^{d}	3.40 ± 0.15^{e}	<.0001

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (%, FA/FA).

[3]∑ 22C HUFA = ∑ 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Pigs fed 1.0% DHA had significantly higher DHA levels in the cerebral cortex, cerebellum and retina than the d1 and MR groups, while d2 pigs showed intermediary levels ($P < 0.0001$; Tables 2.4 and 2.5). DHA accretion in the hippocampus, globus pallidus and inferior colliculus was greatest for pigs fed 1.0% DHA and lowest for the MR group ($P < 0.05$). For these tissues, DHA levels were 17%, 15% and 3% higher, respectively, in the d3 compared with d1 pigs, although differences were not statistically significant.

The sum of the 22-carbon HUFA, corresponding to the sum of 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3 and DHA, was similar across all dietary treatments in all neural tissues reported (Tables 2.4 and 2.5). For these tissues, the magnitude of the increase in non-DHA 22-carbon HUFA was greatest for 22:5n-6. Differences in the composition of 22-carbon HUFA between Diets d1 and d3 were significant and apparent, with d1 pigs having higher 22:5n-6 in cerebral cortex, cerebellum and retina ($P < 0.01$), and higher 22:4n-6 in cerebellum and retina than d3 pigs ($P < 0.001$).

Tissue ARA and DHA dose-response to diet

Figure 2.1 summarizes the linear regression analysis for the tissue ARA dose-response to the dietary ARA level (Diets a1 – a4). ARA levels in the heart and liver were highly responsive to the dietary ARA content ($P < 0.0001$), whereas ARA levels in the cerebral cortex and retina were not esponsive to ARA intake. Regression analysis for the tissue DHA dose-response to dietary DHA content (Diets d1 – d3) is presented in Appendix I (Figure S1). Cerebral cortex, retina, liver and heart DHA levels increased with dietary DHA content ($P < 0.05$). For heart and liver, however, differences were most pronounced between Diets d1 and d3, as increasing the

Table 2.4. FA composition of cerebral cortex, retina and cerebellum from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
Cerebral cortex				*FA (% total FA)*				
ARA	11.95 ± 0.29	11.82 ± 0.60	11.80 ± 0.35	12.04 ± 0.63	11.93 ± 0.40	11.77 ± 0.68	12.33 ± 0.00	NS
22:4n-6	4.03 ± 0.52	4.06 ± 0.16	4.09 ± 0.35	4.21 ± 0.60	4.21 ± 0.32	4.19 ± 0.26	4.81 ± 0.30	NS
22:5n-6	4.49 ± 0.40^{c}	4.44 ± 0.47^{c}	4.68 ± 0.29^{c}	4.61 ± 0.56^{c}	5.52 ± 0.64^{b}	5.49 ± 0.25^{b}	8.26 ± 0.18^{a}	<.0001
22:5n-3	0.20 ± 0.02^{c}	0.19 ± 0.02^{c}	0.19 ± 0.04^{c}	0.19 ± 0.03^{c}	0.20 ± 0.02^{c}	0.25 ± 0.05^{b}	0.32 ± 0.03^{a}	<.0001
DHA	12.10 ± 0.83^{a}	11.91 ± 0.74^{ab}	11.98 ± 0.69^{ab}	11.90 ± 0.69^{ab}	11.30 ± 0.70^{b}	10.56 ± 0.67^{c}	6.88 ± 0.55^{d}	<.0001
∑ 22C HUFA[4]	20.82 ± 0.81	20.60 ± 0.61	20.94 ± 1.01	20.91 ± 0.99	21.23 ± 0.88	20.49 ± 0.79	20.27 ± 0.72	NS
Retina								
ARA	9.48 ± 0.23^{c}	9.70 ± 0.61^{bc}	9.66 ± 0.32^{bc}	9.81 ± 0.31^{b}	9.96 ± 0.37^{b}	9.95 ± 0.25^{b}	10.74 ± 0.50^{a}	0.0004
22:4n-6	1.66 ± 0.15^{e}	1.72 ± 0.12^{de}	1.77 ± 0.12^{d}	1.81 ± 0.17^{d}	1.98 ± 0.19^{c}	2.13 ± 0.12^{b}	2.98 ± 0.17^{a}	<.0001
22:5n-6	1.48 ± 0.30^{d}	1.48 ± 0.32^{d}	1.45 ± 0.16^{d}	1.55 ± 0.30^{d}	2.03 ± 0.64^{c}	2.29 ± 0.26^{b}	5.24 ± 0.39^{a}	<.0001
22:5n-3	0.64 ± 0.04^{a}	0.60 ± 0.05^{b}	0.63 ± 0.06^{ab}	0.60 ± 0.03^{b}	0.55 ± 0.03^{c}	0.60 ± 0.05^{b}	0.60 ± 0.05^{abc}	0.0001
DHA	20.42 ± 1.20^{ab}	20.70 ± 0.54^{a}	20.07 ± 1.47^{ab}	20.49 ± 1.31^{ab}	19.50 ± 1.50^{bc}	18.68 ± 0.96^{c}	14.16 ± 1.37^{d}	<.0001
∑ 22C HUFA	24.29 ± 1.34	24.61 ± 0.49	24.03 ± 1.49	24.55 ± 1.48	24.20 ± 1.31	23.81 ± 0.90	23.10 ± 0.98	NS
Cerebellum								
ARA	10.40 ± 0.30^{c}	10.76 ± 0.26^{bc}	11.03 ± 0.31^{ab}	10.44 ± 0.44^{c}	10.95 ± 0.62^{ab}	10.78 ± 0.43^{bc}	11.48 ± 0.73^{a}	0.007
22:4n-6	3.29 ± 0.41^{d}	3.23 ± 0.38^{d}	3.39 ± 0.32^{cd}	3.43 ± 0.32^{cd}	3.55 ± 0.19^{c}	3.97 ± 0.25^{b}	4.66 ± 0.31^{a}	<.0001
22:5n-6	2.12 ± 0.26^{cd}	2.19 ± 0.34^{d}	2.18 ± 0.19^{cd}	2.17 ± 0.26^{d}	2.70 ± 0.37^{bc}	3.34 ± 1.09^{b}	4.82 ± 0.23^{a}	<.0001
22:5n-3	0.35 ± 0.06^{bc}	0.31 ± 0.05^{cd}	0.32 ± 0.05^{cd}	0.32 ± 0.05^{d}	0.31 ± 0.02^{cd}	0.36 ± 0.05^{b}	0.47 ± 0.04^{a}	<.0001
DHA	11.29 ± 1.61^{a}	11.08 ± 1.68^{a}	11.29 ± 1.19^{a}	11.19 ± 1.44^{a}	10.37 ± 0.75^{ab}	9.83 ± 0.76^{b}	6.10 ± 0.28^{c}	<.0001
∑ 22C HUFA	17.47 ± 1.79	17.19 ± 2.23	17.56 ± 1.51	17.47 ± 1.54	17.36 ± 0.70	17.88 ± 1.37	16.53 ± 0.30	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (%, FA/FA).

[3]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Table 2.5. FA composition of hippocampus, globus pallidus and inferior colliculus from piglets fed varying levels of AA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
Hippocampus				*FA (% total FA)*				
ARA	10.40 ± 0.89	10.78 ± 0.93	10.57 ± 1.09	10.61 ± 1.57	11.10 ± 0.97	10.85 ± 1.23	11.77 ± 0.96	NS
22:4n-6	4.57 ± 0.36	4.68 ± 0.43	4.63 ± 0.57	4.72 ± 0.63	4.78 ± 0.44	5.03 ± 0.51	5.37 ± 0.43	NS
22:5n-6	2.89 ± 0.22^{c}	2.96 ± 0.39^{c}	3.06 ± 0.39^{cd}	2.68 ± 0.67^{d}	3.71 ± 0.40^{b}	3.31 ± 0.53^{bc}	5.58 ± 0.60^{a}	<.0001
22:5n-3	0.24 ± 0.03^{ab}	0.22 ± 0.05^{bc}	0.22 ± 0.02^{bc}	0.20 ± 0.05^{bc}	0.19 ± 0.02^{c}	0.24 ± 0.04^{ab}	0.27 ± 0.04^{a}	0.009
DHA	9.89 ± 0.26^{a}	9.74 ± 1.43^{a}	9.66 ± 0.68^{ab}	9.63 ± 1.73^{a}	9.44 ± 1.03^{a}	8.27 ± 1.48^{b}	6.12 ± 0.68^{c}	0.0002
∑ 22C HUFA[4]	17.20 ± 1.41	17.60 ± 1.87	17.13 ± 1.99	17.23 ± 2.89	18.12 ± 1.44	16.84 ± 2.23	17.34 ± 0.64	NS
Globus pallidus								
ARA	10.33 ± 0.68	9.33 ± 1.28	10.07 ± 0.44	9.92 ± 0.76	10.02 ± 0.88	10.09 ± 0.96	10.73 ± 0.82	NS
22:4n-6	3.81 ± 0.32^{bc}	3.59 ± 0.73^{b}	4.25 ± 0.51^{ab}	3.98 ± 0.87^{bc}	3.72 ± 0.37^{bc}	4.04 ± 0.58^{bc}	4.78 ± 0.65^{a}	0.02
22:5n-6	2.93 ± 0.37^{b}	2.24 ± 0.77^{c}	2.81 ± 0.40^{bc}	2.64 ± 0.58^{bc}	3.16 ± 0.73^{b}	3.04 ± 0.78^{b}	5.40 ± 0.92^{a}	<.0001
22:5n-3	0.15 ± 0.12	0.12 ± 0.04	0.13 ± 0.03	0.13 ± 0.02	0.11 ± 0.04	0.14 ± 0.06	0.19 ± 0.06	NS
DHA	9.45 ± 0.97^{a}	8.09 ± 2.61^{ab}	8.80 ± 0.94^{ab}	8.18 ± 1.36^{ab}	7.91 ± 1.95^{b}	7.66 ± 1.68^{bc}	6.08 ± 0.35^{c}	0.01
∑ 22C HUFA	16.74 ± 1.12	14.49 ± 3.72	16.44 ± 1.37	15.41 ± 2.20	15.37 ± 2.68	15.34 ± 2.50	16.92 ± 1.43	NS
Inferior colliculus								
ARA	8.58 ± 0.35	8.55 ± 0.75	8.85 ± 0.84	8.61 ± 0.52	8.63 ± 0.50	8.97 ± 0.73	9.45 ± 1.25	NS
22:4n-6	3.54 ± 0.21^{b}	3.36 ± 0.19^{b}	3.55 ± 0.31^{b}	3.56 ± 0.29^{b}	3.42 ± 0.30^{b}	3.70 ± 0.35^{b}	4.68 ± 1.16^{a}	<.0001
22:5n-6	1.58 ± 0.49^{bc}	1.50 ± 0.35^{c}	1.60 ± 0.45^{bc}	1.62 ± 0.52^{bc}	1.93 ± 0.53^{b}	1.91 ± 0.32^{b}	3.76 ± 0.45^{a}	<.0001
22:5n-3	0.19 ± 0.02^{ab}	0.18 ± 0.04^{abc}	0.19 ± 0.04^{ab}	0.15 ± 0.02^{c}	0.16 ± 0.03^{bc}	0.18 ± 0.03^{bc}	0.21 ± 0.06^{a}	0.02
DHA	10.71 ± 0.94^{a}	10.32 ± 1.88^{a}	10.84 ± 1.50^{a}	9.58 ± 1.21^{a}	10.18 ± 1.27^{a}	10.53 ± 0.85^{a}	6.27 ± 1.14^{b}	<.0001
∑ 22C HUFA	16.35 ± 1.10	15.66 ± 2.18	16.46 ± 2.03	15.28 ± 1.60	16.02 ± 1.46	16.61 ± 1.23	15.40 ± 1.74	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (%, FA/FA).

[3]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

DHA content from 0.62 to 1.0% total FA (Diets d2 vs. d3) did not significantly increase heart or liver DHA levels. Cerebellum and hippocampus DHA levels also increased with dietary DHA content ($P < 0.05$), while globus pallidus and inferior colliculus DHA levels did not correlate with dietary DHA content (Figure S2, Appendix I).

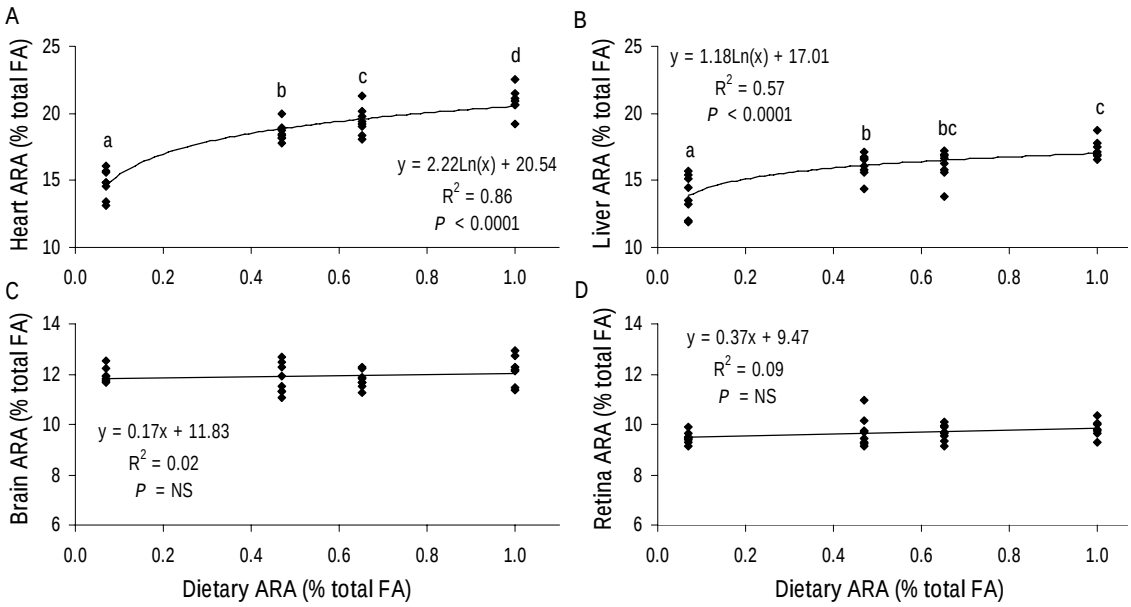

Figure 2.1. ARA tissue dose-response to dietary ARA with constant DHA (1.0% total FA). Dietary ARA content for a1, a2, a3 and a4 was 0.09, 0.53, 0.69 and 1.06% total FA, respectively. (A) Heart and (b) Liver responded significantly ($P < 0.0001$) while (C) Brain (cerebral cortex) and (D) Retina did not. Each point represents one animal (n = 32 per panel). $P < 0.05$ indicates significant correlation between dietary ARA content and tissue ARA level; NS, not significant. Student's t-test used to determine significance of pairwise comparisons of mean tissue ARA content; means not sharing a common letter are significantly different ($P < 0.05$).

ARA and DHA competition for tissue incorporation

Dietary ARA level had no effect on DHA accretion in the heart, liver or neural tissues ($P > 0.05$) (Figure 2.2; Figure S3, Appendix I). Conversely, there was an apparent competition between liver ARA and dietary-derived

DHA (Diets d1 – d3), with d3 pigs showing lower liver ARA levels than d1 pigs (P = 0.004) (Figure 2.3). This competition was not detected in the heart, brain or retina, indicating that the dietary DHA content has no effect on ARA levels in these tissues (Figure 2.3).

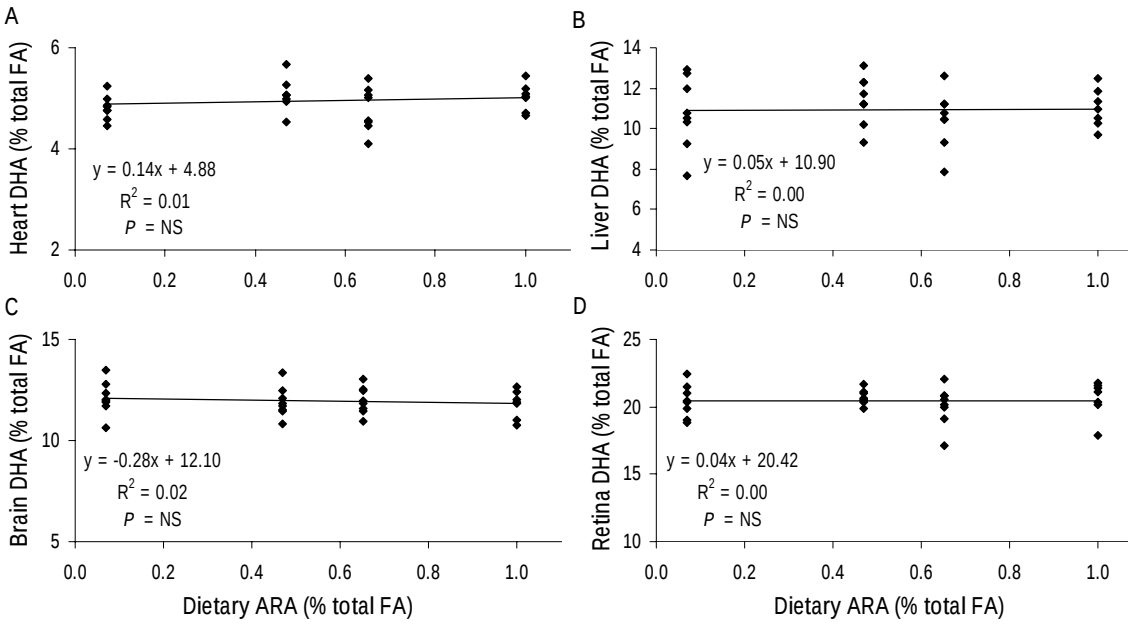

Figure 2.2. DHA tissue dose-response to dietary ARA content with constant DHA (1.0% total FA). Dietary ARA content for a1, a2, a3 and a4 was 0.09, 0.53, 0.69 and 1.06% total FA, respectively. Increasing ARA had no effect on DHA levels in (A) Heart, (B) Liver, (C) Brain (cerebral cortex) or (D) Retina. Each point represents one animal (n = 24 per panel). P < 0.05 indicates significant correlation between dietary ARA content and tissue DHA level; NS, not significant. Student's t-test used to determine significance of pairwise comparisons of mean tissue DHA content; means not sharing a common letter are significantly different (P < 0.05).

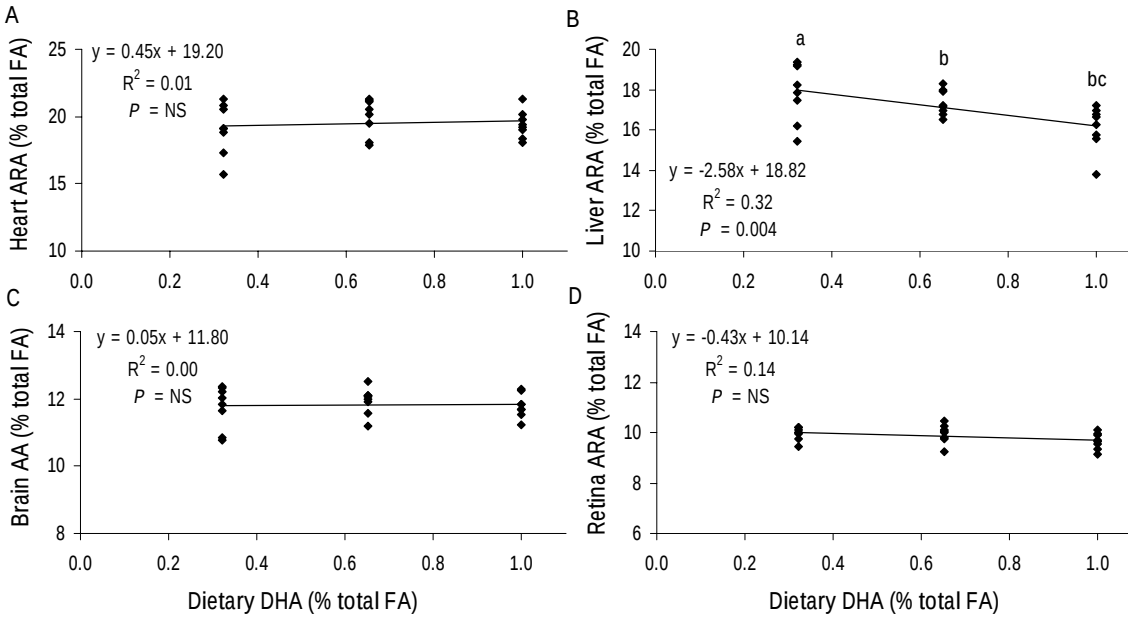

Figure 2.3. ARA tissue dose-response to dietary DHA content with constant ARA (0.67% total FA). Dietary DHA content for d1, d2 and d3 was 0.32, 0.62 and 1.01% total FA, respectively. Increasing DHA reduced ARA levels in (B) Liver but not (A) Heart, (C) Brain (cerebral cortex) or (D) Retina. Each point represents one animal (n = 24 per panel). $P < 0.05$ indicates significant correlation between dietary DHA content and tissue ARA level; NS, not significant. Student's t-test used to determine significance of pairwise comparisons of mean tissue ARA content; means not sharing a common letter are significantly different ($P < 0.05$).

Discussion

The neonatal pig serves as a practical biomedical model of human infant development. Pigs are similar to humans in their metabolic responses, genetics of the FA desaturases and rates of perinatal brain growth (28,29). Their rapid postnatal growth enables facile collection of pre-clinically relevant data over a relatively short study duration.

The sensitivity of heart ARA to the dietary ARA has been reported from time to time in pigs (19,21) and rats (17,18), yet it is poorly understood on a mechanistic basis and its clinical significance is unknown. In the present study, heart ARA levels reflected dietary ARA content and were significantly

different for pigs in all ARA groups including those with intermediate intakes, a2 (18.6 ± 0.7% total FA) and a3 (19.4 ± 1.0% total FA), indicating that heart ARA is limited by intake, at least at high DHA levels. These observations are particularly relevant to ARA recommendations for infant formula as 0.53% (a2) is slightly above the worldwide ARA mean in human breast milk and 0.67% (a3) is the level currently added in formula and is near the high end of breast milk values reported. ARA serves several known functions in cardiac physiology, such as providing precursor for eicosanoid signaling molecules and acting directly to modulate voltage-gated ion channel activity and cellular excitability (30). The degree to which heart ARA status influences cardiac physiology in the developing neonate, as well as the potential long term implications, remains to be determined.

Biosynthesis of ARA and DHA from the nutritionally essential linoleic and linolenic acids occurs through a series of elongation and desaturation reactions catalyzed by a common set of enzymes (31). Studies with humans indicate that the rate of postnatal endogenous DHA synthesis is insufficient to support DHA accretion in neural tissues (32,33), and results in poorer visual and cognitive performance compared with infants consuming preformed DHA (3,5,6). These observations provide the basis for the addition of DHA to formula. Rates of endogenous synthesis of ARA from linoleic acid appear sufficient to maintain brain ARA levels in adult rats (34) but remain to be thoroughly investigated in growing animals. In the present study, higher levels of dietary ARA correlated with increased heart and liver ARA, while brain ARA was constant and unaffected by ARA intake. The trend for a modest increase in retina ARA with increasing dietary ARA was not supported by the linear regression analysis for the ARA dose-response. Liver lipids typically reflect

dietary FA content but are also influenced by the expression and regulation of enzymes involved in LCPUFA biosynthesis (31,35). The heart, too, expresses the FA desaturases and elongase enzymes (Elovl-1, -5 and -6) capable of synthesizing ARA, but is not generally considered a major site of LCPUFA biosynthesis (36). The sensitivity of the heart to dietary ARA level therefore suggests that the ARA content is influenced predominantly by preferential FA uptake and incorporation. Further, these data indicate that the rate of endogenous ARA biosynthesis may not be sufficient to match ARA accretion in the growing the heart.

The choice to include an MR reference group provided a unique opportunity to investigate the contribution of sow milk, essentially devoid of DHA but not ARA, to the LCPUFA status of the developing offspring. This treatment is not unlike an extreme case of DHA-deficient breast milk that is reflective of few human populations worldwide (2,37). Because of this, we advise caution in extending results observed with the MR group, in particular comparing MR pigs with the FR groups fed higher levels of DHA, to breastfeeding practices in humans.

The competition between n-3 and n-6 for tissue incorporation and the resulting effect on LCPUFA biosynthesis is well-studied. The relative availability of linoleic and linolenic acids, as well as their LCPUFA derivatives, drives the biosynthesis of n-3 and n-6 LCPUFA and modulates tissue levels of the two FA families in a reciprocal manner (20,38,39). Over the range of dietary ARA levels in the present study, tissue DHA was constant and unaffected by diet. On the other hand, we observed a specific reduction in liver ARA with the highest DHA intakes, consistent with previous reports in pigs (19) and baboon neonates (23) consuming preformed DHA. These

observations reinforce the concept that ARA and DHA in the liver are closely linked to diet because of the liver's central role in LCPUFA biosynthesis, and are specific to function in the heart and neural tissue; all tissues being modulated by genetic and extra-dietary factors.

The sensitivity of central nervous tissue DHA level has been previously investigated in numerous studies with term and preterm neonatal baboons (22,23,40). The brain consistently shows increased region-specific DHA accretion related to level and duration of preformed DHA feeding, and prematurity appears important. Here we observed higher levels of DHA in all tissues with increasing DHA content. Pigs in the high DHA group showed significantly higher levels of DHA in the cerebral cortex, retina and cerebellum compared with pigs in the low DHA group. Increases in mean DHA were 13%, 7% and 15%, respectively for these neural tissues. DHA levels in the hippocampus, globus pallidus and inferior colliculus were 17%, 15% and 3% higher in the high DHA group compared with the low DHA group; while these differences were not statistically significant they may be of biological importance. The relative insensitivity of basal ganglia, limbic and midbrain regions to postnatal dietary DHA consumption, compared with the cerebral cortex, is consistent with Hsieh et al. (23), and suggests that these deep brain regions may be influenced to a greater extent by the DHA status of the mother during gestation. Thus, these data support the recent dietary recommendations on total fat and fatty acids by the FAO/WHO for the provision of 200 mg/day DHA in the diets of pregnant and lactating women (41).

Pigs that exhibited the lowest neural tissue DHA also showed enriched tissue 22:4n-6, 22:5n-6 and 22:5n-3, with greatest increases occurring in

22:5n-6. The increase in tissue 22:5n-6 in animals fed n-3-inadequate diets is well-characterized (42-44) and presumably occurs as an alternate substrate source for DHA-dependent functions in neural tissue. Recent examination of the frontal cortex from rat pups fed an n-3-deficient diet revealed a highly specific substitution of 22:5n-6, but not 22:4n-6 or ARA for DHA in brain phosphatidyl ethanolamine and phosphatidyl serine (45). Tight regulation of the "reciprocal replacement" of DHA and 22:5n-6 (42) among brain phospholipid molecular species illustrates the essential structure-function relationship between DHA and outcomes such as vision or cognition. Functionally, low DHA levels in neural tissue cannot be fully compensated by increased 22:5n-6 and manifests as poorer electroretinogram performance (46) and spatial learning (47). In the present study, the composition difference in neural tissue 22-carbon HUFA between diets d1 and d3 is of particular interest. Pigs in the low DHA group had significantly lower DHA and higher 22:5n-6 in cerebral cortex, cerebellum and retina. DHA at 0.32% total FA is the level currently added to infant formulas. Higher levels may provide further benefit for visual acuity and cognition, especially in target populations with endogenously low DHA status (5,9,11). Importantly, the d1-d3 groups held ARA at 0.67% which is at the highest levels found in human breast milk (2), and similar to the MR group which had extremely low DHA (Table 2.2).

Changes in tissue FA composition could influence markers of formula safety and measures of overall development. The dietary ARA level (0.09 – 1.0% total FA) had a negligible effect on safety as assessed by clinical chemistry and measures of immune function in our piglets fed 1.0% DHA (Chapter One). Likewise, there were no differences in growth among them, and no reported differences in vigor by those observing piglets daily.

In summary, results from the present study demonstrate a tissue-specific responsiveness to the dietary ARA level in rapidly growing neonatal pigs. Whereas brain ARA remained unaffected by diet ARA, heart and liver ARA levels were responsive. Dietary ARA level had no effect on DHA accretion in heart, liver or neural tissue. The magnification of heart ARA levels with increasing dietary ARA content suggests that the growing heart specifically incorporates and concentrates ARA at a rate that may exceed the biosynthetic capacity for ARA. Further investigations are warranted to determine the biological implication of heart ARA status in the developing neonate.

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CHAPTER THREE

EVALUATION OF BIOEQUIVALENCY AND TOXICOLOGICAL EFFECTS OF THREE SOURCES OF ARACHIDONIC ACID (ARA) IN DOMESTIC PIGLETS*

Abstract

Arachidonic acid (ARA) is routinely added to infant formula to support growth and development. We evaluated the safety and bioequivalence of three oils providing ARA for potential use in infant formula using the neonatal pig model. On d 3 of age, domestic pigs were matched for weight and gender and assigned to 1 of 3 formula diets, each containing ARA at 0.62 – 0.67% total fatty acids. Long chain polyunsaturated fatty acids (LCPUFA) in the Control diet were provided by ARASCO and DHASCO (Martek Biosciences, Corp.) in a formulation similar to Enfamil; ARA in experimental diets (a1 and a2) came from proprietary blends of oils. Blood collection was performed weekly and body weight measurements taken every 2-3 d. Pigs were sacrificed on d 22 and organs sampled. Intake and growth was similar across all diets. Few differences in clinical chemistry and hemogram parameters were detected, with aspartate aminotransferase and creatine kinase levels highest in the Control. Liver sections had no signs of histopathological abnormality. ARA and docosahexaenoic acid (DHA) levels in cerebral cortex, retina and heart were not different across all diets. In the liver, ARA levels

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were 2% lower in a1 pigs compared with the Controls (P = 0.002) while DHA levels in the a1 pigs averaged 7% higher than the Control and a2 groups (P < 0.05). We conclude that the experimental ARA oils are safe and bioequivalent to a commercially available ARA source in the domestic piglet.

Introduction

Arachidonic acid (ARA) is a long chain polyunsaturated fatty acid (LCPUFA) that is routinely added to infant formula along with docosahexaenoic acid (DHA). Both are natural components of breast milk and are important for growth and development during the perinatal period (1-3). ARA and DHA serve as the major LCPUFA in central nervous tissue and in the heart ARA comprises upwards of 25% of total fatty acids (FA) (Chapter Two). Tissue ARA and DHA accretion during growth occurs via the uptake of preformed LCPUFA from breast milk or formula or through the endogenous biosynthesis of LCPUFA from the dietary essential FA, linoleic and linolenic acids. Rates of endogenous ARA and DHA biosynthesis appear to be a limiting factor in the accretion of LCPUFA in tissues (4-6)(Chapter Two), indicating a requirement for these LCPUFA in the diets of human infants. In 1994, the FAO/WHO set recommendations for ARA and DHA in the formula of term infants at 0.66% and 0.33% of total FA, respectively (7). These levels are based largely on mean worldwide breast milk levels (8,9) and supported by the evidence for benefit in vision and cognitive performance in clinical studies with human infants (10,11).

Single-cell oils serve as a convenient vehicle for adding target levels of LCPUFA to commercial infant formula. In the U.S., two main sources of ARA have been determined to be Generally Regarded as Safe (GRAS) for use in

infant formula, ARASCO (Martek Biosciences, Corp., Columbia, MD) (12,13) and SUNTGA40S (Suntory, Ltd., Osaka, Japan) (14). Both are triglyceride oils derived from the fungus *Mortierella alpina* (*M. alpina*) and contain ARA at approximately 40% total FA. A third oil, "refined arachidonic acid-rich oil" (RAO; Cargill, Inc., Wuhan, China) also derived from *M. alpina* is currently pending FDA approval for use in preterm and term infant formulas (15). These ARA oils may be derived from a common fungal source but differences in the manufacturing process inevitably lead to subtle variations in the final product. Of particular importance here are the levels of trace chemical and fat soluble components, and potentially bioactive microbiological constituents. While an ARA oil may comprise less than 2% of the total fat in formula, the extended consumption of these oils by the vulnerable, growing infant population may exaggerate any toxicological or allergenic effect caused by trace or undetected contaminants (16,17). Thus, preclinical feeding studies with animals are essential to determine the safety of novel formulations for use in the diets of human infants.

The present study sought to determine the safety and bioequivalence of two experimental, proprietary ARA oils for potential use in infant formula compared with the commercially available ARASCO using the neonatal pig model. Piglets were fed one of three ready-to-use formulas that provided ARA at 0.64% and DHA at 0.34% of total FA from day 3 to 22 of life, upon which tissues were analyzed for ARA and DHA accretion and livers examined for pathohistological changes. We hypothesized that the experimental ARA oils would be safe, nutritionally bioequivalent to ARASCO and produce no toxicological effects in our neonatal pigs.

Materials and methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Cornell University. Domestic piglets were selected for gender and weight from 5 sows at the swine facility at Cornell University. One day before the scheduled, term farrowing date, pregnant sows were injected with 2 cc of Lutalyse (Pfizer Animal Health, Kalamazoo, MI) to induce farrowing and obtain a block of piglets with the same birth date. Newborn piglets were processed according to standard facility practices (i.e. intramuscular injection of iron dextran (100 mg) and penicillin G (½ cc), tooth clip, tail dock, ear notch) and remained with the sow until day 3 of age, upon which they were matched for weight and assigned to one of three experimental formula diets (n=8 per diet). Piglets were then transported to the campus large animal research facility where they were housed individually in raised metal cages, provided appropriate enrichment and maintained on a 16/8 hr light/dark cycle.

Diets

Experimental formulas were designed to meet or exceed nutrient requirements for growing pigs 3 – 5 kg in body weight (18). Milk replacer diets were prepared as ready-to-use formulas that provided 1.0 kcal/ml with a target nutrient composition as follows (% wt/wt): protein, 4.9; fat, 5.5; ash, 1.2. The detailed composition and nutrient analysis for the experimental formulas is presented in Table 3.1. Target specifications for ARA and DHA were 35.8 and 17.9 mg/100 kcal. LCPUFA in the Control diet were supplied by ARASCO and DHASCO (Martek Biosciences, Corp., Columbia, MD) in a formulation similar

Table 3.1. Nutrient composition of experimental diets

Nutrient (% wt/wt)[1]	Control	a1	a2
Fat	5.5	5.6	5.6
Protein	4.9	4.9	4.8
Carbohydrate	6.4	6.1	6.2
Ash	1.2	1.2	1.2
Total solids	18.1	17.7	17.8
Mineral (mg/100 kcal)			
Na	59.3	62.6	60.6
Mg	15.0	14.5	14.6
P	146.0	149.7	146.3
K	306.3	301.9	304.4
Ca	217.5	214.8	208.8
Vitamin (units/100 kcal)			
Vitamin E (IU)	1.6	1.6	1.6
Vitamin K (mcg)	16.7	18.3	17.5
Vitamin D (IU)	13.4	13.2	13.5
Vitamin C (mg)	6.3	6.7	6.3
Vitamin A (IU)	83.0	88.0	83.0
Thiamine HCl (mcg)	82.8	67.1	66.8
Riboflavin (mcg)	204.5	185.7	188.3
Vitamin B6 (mcg)	65.6	60.5	69.4
Vitamin B12 (mcg)	0.5	0.4	0.5

[1]Composition taken as percent of total weight (wt).

Table 3.2. FA composition of experimental diets[1]

Diet	Control	a1	a2
	FA (% total FA)		
10:0	0.70 ± 0.02	0.74 ± 0.02	0.46 ± 0.01
12:0	8.19 ± 0.08	8.12 ± 0.09	7.37 ± 0.07
14:0	4.21 ± 0.04	4.24 ± 0.03	4.20 ± 0.03
16:0	21.76 ± 0.05	21.77 ± 0.08	22.07 ± 0.09
16:1	0.18 ± 0.01	0.18 ± 0.01	0.19 ± 0.02
18:0	4.58 ± 0.02	4.50 ± 0.02	4.53 ± 0.04
18:1	38.14 ± 0.12	37.96 ± 0.15	38.59 ± 0.15
18:2n-6	17.74 ± 0.03	17.86 ± 0.06	17.96 ± 0.04
Conjugated 18:2	0.29 ± 0.03	0.31 ± 0.01	0.25 ± 0.03
18:3n-6	0.10 ± 0.01	0.11 ± 0.00	0.10 ± 0.01
18:3n-3	2.00 ± 0.03	2.01 ± 0.04	2.01 ± 0.03
20:0	0.35 ± 0.01	0.35 ± 0.00	0.36 ± 0.00
20:1	0.21 ± 0.01	0.21 ± 0.01	0.21 ± 0.01
20:2n-6	0.03 ± 0.00	0.01 ± 0.01	0.03 ± 0.00
20:3n-6	0.07 ± 0.00	0.08 ± 0.01	0.08 ± 0.00
ARA	0.67 ± 0.01	0.62 ± 0.00	0.67 ± 0.00
22:0	0.27 ± 0.01	0.30 ± 0.01	0.32 ± 0.01
DHA	0.35 ± 0.01	0.35 ± 0.01	0.34 ± 0.01
23:0	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.00
24:0	0.12 ± 0.00	0.21 ± 0.01	0.22 ± 0.01
∑ SFA[2]	40.18 ± 0.15	40.22 ± 0.20	39.53 ± 0.17
∑ MUFA	38.53 ± 0.13	38.36 ± 0.15	38.99 ± 0.14
∑ n-6	18.62 ± 0.03	18.67 ± 0.06	18.83 ± 0.04
∑ n-3	2.34 ± 0.04	2.36 ± 0.03	2.35 ± 0.04
18:2n-6/18:3n-3	8.89 ± 0.15	8.91 ± 0.18	8.94 ± 0.16

[1]Values represent means ± SD, n = 3 extractions per diet.

[2]SFA, saturated FA; MUFA, monounsaturated FA.

to that currently used in the commercial human infant formula Enfamil (Mead-Johnson Nutrition, Evansville, IN). LCPUFA in the experimental diets, a1 and a2 were provided by a proprietary blend of oils that may include SUNTGA40S, RAO or a novel ARA source. The detailed FA composition of the experimental formulas is presented in Table 3.2. Fresh formula was provided three times per day in stainless steel bowls fixed into the cage doors and water was offered ad libitum. Piglets were fed at 80% ad libitum intake, based on pilot data, and formula intakes were recorded daily.

Sampling

Body weights measurements were taken every other day during the first week of life and thereon every third day for the remainder of the study. Non-fasted blood samples were collected from the anterior vena cava into EDTA-containing vacutainer tubes (BD, Franklin Lakes, NJ) on days 3, 7, 14 and 21 of age. Whole blood was separated into plasma, red blood cell (RBC) and white cell fractions using Leucosep tubes (Grenier Bio-One, Monroe, NC) with lymphocyte separation media (Mediatech, Inc., Manassas, VA) according to manufacturer's directions. Piglets were sacrificed on day 22 of age via an intravenous injection of Fatal Plus (1 ml/4.54 kg body weight; Vortech Pharmaceuticals, Dearborn, MI, USA) followed by exsanguination. Organs were harvested, weighed and samples flash frozen in liquid nitrogen within 10 minutes of cessation of heart beat.

Clinical chemistry and hemogram analysis

Plasma and EDTA-blood from piglets on day 21 of age were delivered promptly to the Animal Health Diagnostic Center (College of Veterinary

Medicine, Cornell University) for analysis of clinical chemistry and hemogram parameters.

Liver histopathology

Freshly harvested samples from the right central lobe (~ 1 g) were fixed in 10% neutral buffered formalin and submitted to the Pathohistology Laboratory (College of Veterinary Medicine, Cornell University) where they were embedded in wax, sectioned and stained with hematoxylin and eosin. Sections were examined under light microscope by a veterinary pathologist for determination of histopathological abnormalities.

Fatty acid analysis

FA methyl esters (FAME) were prepared from approximately 50 mg tissue and 50 µl of plasma, RBC and formula according to the One-step hydrolysis, extraction and methylation procedure (19) with modifications (20). A detailed protocol for the One-step method is presented in Appendix II. Retinas, which were suspended in saline at necropsy, were first dried using a Savant SpeedVac Concentrator (Thermo Fisher Scientific, Waltham, MA). FAME were quantified on a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a BPX70 fused silica column (25 m x 0.22 mm i.d. x 0.25 µm film; SGE Incorportated, Austin, TX) and integrated using PeakSimple 3.78 software (SRI Instruments, Torrance, CA). An equal weight FAME mixture was used daily to verify response factors. FAME were structurally identified by covalent-adduct chemical ionization mass spectrometry on a Saturn 2000 mass spectrometer (Varian, Inc., Walnut Creek, CA) attached to a Varian Star 3400 gas chromatograph (21).

Statistical analysis

Statistical analysis was carried out using the Fit Model platform of JMP (2008 SAS Institute, 8.0) to fit mixed models. Fixed effects were diet, gender, day 3 of age body weight and the full factorial of interactions. Interaction effects were considered significant at $P < 0.10$ and fixed effects at $P < 0.05$. For each parameter analyzed, effects not considered significant were removed from the final model. Random effects were litter and animal nested within litter for repeated measures of body weight and RBC FA content. Significance of pairwise comparisons was determined using the Student's t-test. Values are reported as means ± SD. Linear regression analysis was performed to determine the relationship between RBC LCPUFA content and postnatal age.

Results

Formula composition

Total formula intakes averaged 29.6 ± 1.7 L and were similar for all three dietary treatment groups. The experimental formulas provided 1 kcal/ml, corresponding to a mean total energy intake of 29,600 ± 1,700 kcal per pig and a mean daily intake of 1.5 L or 1,500 kcal. Mean total intake of ARA was 10.60 ± 0.59 g, while the mean total intake of DHA was 5.30 ± 0.30 g.

Clinical observations and growth

All 24 piglets remained on the study until day 21 of age, and very few health ailments were noted during the course of the study. Loose stools during the first week of life were the greatest concern, although by the second week of life, the occurrence of loose stools had cleared in most piglets. One piglet was given an intramuscular injection of penicillin G benzathine (75,000

IU) by the attending veterinarian on day 11 of age for an undiagnosed, yet persistent disinterest in consuming formula. That animal grew significantly less than all other pigs starting on day 13 of age, as determined using Grubb's test for statistical outliers, and was removed from the body weight analysis starting at this time point.

The temporal pattern of body weight gain during the study is presented in Figure 3.1. Piglets weighed 2.1 ± 0.2 kg at the start of the study (day 3 of age) and rapidly grew to 8.4 ± 0.4 kg by day 22 of age. Mean body weights were similar among the three dietary treatments at every time point measured. Organ weights at necropsy, both relative and absolute, were similar among all dietary treatment groups. Figure 3.2 presents the summary of relative organ weights (organ weight as a percentage of body weight at necropsy) for piglets sacrificed on day 22 of age.

Hemogram and clinical chemistry

A summary of mean hemogram and clinical chemistry parameters is presented in Table 3.3. Only two parameters, aspartate aminotransferase (AST) and creatine kinase, were determined to be significantly influenced by diet. Control pigs had significantly higher AST (37 ± 13 U/L) than a1 (28 ± 7 U/L) and a2 (25 ± 7 U/L) pigs (P = 0.01), and higher creatine kinase (895 ± 520 U/L) than the a1 (541 ± 227 U/L) and a2 (520 ± 228 U/L) pigs (P = 0.001). AST and creatine kinase levels were similar between the a1 and a2 groups. Additional parameters that were unaffected by diet were mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red cell distribution width, segmented neutrophils, monocytes, eosinophils, basophils,

large unstained cells, mean platelet volume, creatinine, phosphate and total bilirubin (data not presented).

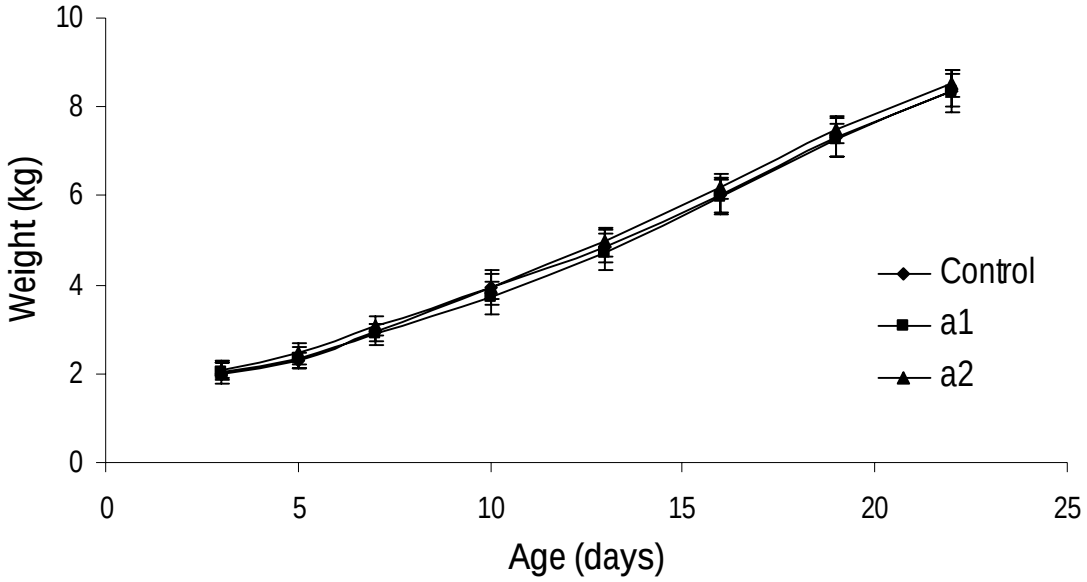

Figure 3.1. Temporal pattern of body weight gain in piglets from day 3 to 22 of age. Values represent means ± SD, n = 8/diet. One piglet from Diet a1 was not included in body weight measurements on days 13 – 22 due to a slower rate of growth caused by an undiagnosed, yet persistent disinterest in formula consumption. Body weights were similar among all dietary treatment groups at every time point measured.

Liver histopathology

Histological diagnosis: Normal. "*Comment*: All samples were within normal histopathological limits. The lobular architecture was intact, the portal regions were unremarkable and there was no evidence of cholestasis. Hepatocytes were uniform in size and shape, replete with glycogen and contained no discernable lipid. No mitrotic figures were observed and only rare binucleate hepatocytes were noted. Occasional Kupffer cells were present but no hepatic stellate (Ito) cells could be identified. Variable numbers of small foci of extramedullary hematopoietic tissue were randomly dispersed

throughout the parenchyma (incidental). No histopathological changes were present in the sections examined. In particular, no changes similar to those described in rodent studies of peroxisome proliferator exposure were detected."

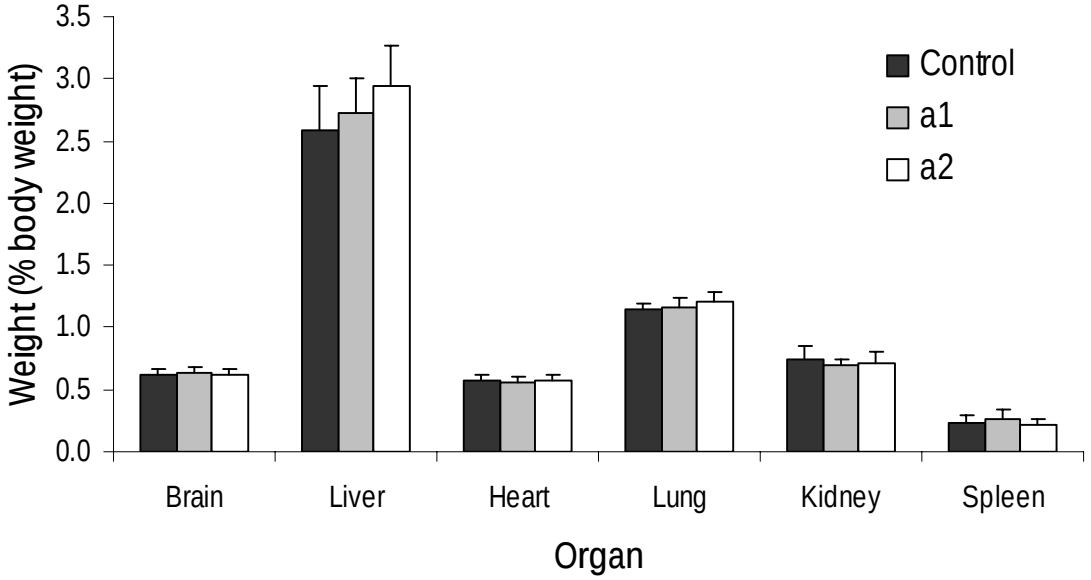

Figure 3.2. Relative organ weights from piglets on day 22 of age. Values represent means ± SD, n = 8 for Control and Diet a2; n = 7 for Diet a1. Pairwise comparisons determined using Student's t-test. Organ weights were similar for all dietary treatment groups.

Tissue LCPUFA accretion

The FA composition of heart, liver, brain (cerebral cortex) and retina, harvested from pigs on day 22 of age is presented in Tables 3.4 and 3.5. Mean ARA levels in the brain, retina and heart were 10.97 ± 0.36%, 10.50 ± 0.43%, 20.38 ± 0.82% of total FA, respectively, and were similar for all three dietary treatment groups. ARA levels in the liver were 2% lower in pigs fed Diet a1 (17.33 ± 0.78% FA) compared with the Control (17.66 ± 0.49% FA), while the a2 pigs showed an intermediary liver ARA content (17.38 ± 0.57%

Table 3.3. Summary of hemogram and clinical chemistry parameters from piglets on day 21 of age[1-2]

Test	Control	a1	a2	*P*
Hematocrit (%)	43 ± 3	46 ± 5	42 ± 2	NS
Hemoglobin (g/dL)	13.2 ± 0.9	13.8 ± 1.3	12.9 ± 0.5	NS
RBC (mill/μL)	6.3 ± 0.4	6.5 ± 0.5	6.3 ± 0.2	NS
WBC (thou/μL)	10.5 ± 2.2	10.9 ± 1.3	9.9 ± 2.6	NS
Lymphocytes (thou/μL)	6.1 ± 1.1	6.4 ± 1.1	5.7 ± 1.3	NS
Platelet Count (thou/μL)	709 ± 76	697 ± 169	667 ± 136	NS
Urea (mg/dL)	8 ± 2	8 ± 1	7 ± 2	NS
Creatinine (mg/dL)	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	NS
Total Protein (g/dL)	4.6 ± 0.3	4.7 ± 0.2	4.5 ± 0.2	NS
Albumin (g/dL)	3.6 ± 0.3	3.5 ± 0.2	3.5 ± 0.1	NS
Globulin (g/dL)	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.1	NS
Glucose (mg/dL)	178 ± 37	165 ± 31	166 ± 33	NS
AST (U/L)	37 ± 13[a]	28 ± 7[b]	25 ± 7[b]	0.01
GGT (U/L)	23 ± 11	24 ± 14	24 ± 16	NS
Creatine Kinase (U/L)	895 ± 520[a]	541 ± 227[b]	520 ± 228[b]	0.001

[1]Abbreviations: RBC, red blood cell count; WBC, white blood cell count; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

[2]Values represent means ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

Table 3.4. FA composition of heart and liver from piglets fed one of three sources of ARA[1]

Diet	Control	a1	a2	*P*
Heart		*FA (% total FA)*		
Σ SFA[2]	31.92 ± 0.29	32.15 ± 0.49	32.37 ± 0.39	NS
Σ MUFA	18.53 ± 0.75	18.35 ± 0.77	18.09 ± 0.74	NS
ARA	20.67 ± 0.56	19.93 ± 0.79	20.57 ± 0.93	NS
22:4n-6	0.92 ± 0.10	0.86 ± 0.09	0.83 ± 0.13	NS
22:5n-6	*tr.*	*tr.*	*tr.*	n/a
Σ n-6	43.40 ± 0.86	43.44 ± 1.12	43.59 ± 1.00	NS
20:5n-3	0.42 ± 0.04	0.39 ± 0.03	0.39 ± 0.04	NS
22:5n-3	0.76 ± 0.08	0.71 ± 0.04	0.73 ± 0.05	NS
DHA	4.16 ± 0.39	4.04 ± 0.28	3.98 ± 0.35	NS
Σ n-3	6.05 ± 0.40	5.95 ± 0.31	5.85 ± 0.40	NS
ARA/DHA	5.00 ± 0.38	4.96 ± 0.40	5.22 ± 0.63	NS
Σ 22C HUFA[3]	5.84 ± 0.37	5.61 ± 0.34	5.53 ± 0.37	NS
Liver				
Σ SFA	42.15 ± 0.91	42.46 ± 0.57	41.89 ± 0.74	NS
Σ MUFA	13.64 ± 1.17	13.50 ± 0.71	13.89 ± 1.24	NS
ARA	17.66 ± 0.49[a]	17.33 ± 0.78[b]	17.38 ± 0.57[ab]	0.01
22:4n-6	0.64 ± 0.07	0.63 ± 0.10	0.65 ± 0.08	NS
22:5n-6	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.02	NS
Σ n-6	34.10 ± 0.69	33.49 ± 0.59	34.17 ± 0.55	NS
20:5n-3	0.33 ± 0.04	0.28 ± 0.08	0.31 ± 0.05	NS
22:5n-3	1.03 ± 0.18	1.04 ± 0.13	1.04 ± 0.06	NS
DHA	7.70 ± 0.47[b]	8.23 ± 0.38[a]	7.62 ± 0.62[b]	0.046
Σ n-3	9.80 ± 0.47	10.21 ± 0.43	9.70 ± 0.56	NS
ARA/DHA	2.30 ± 0.12	2.11 ± 0.12	2.29 ± 0.15	NS
Σ 22C HUFA	9.43 ± 0.52	9.96 ± 0.33	9.37 ± 0.68	NS

[1]Values represent means ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]SFA, saturated FA; MUFA, monounsaturated FA.

[3]∑ 22C HUFA = ∑ 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Table 3.5. FA composition of brain (cerebral cortex) and retina from piglets fed one of three sources of ARA[1]

Diet	Control	a1	a2	*P*
Cerebral cortex	*FA (% total FA)*			
∑ SFA[2]	44.49 ± 0.58	44.82 ± 0.74	44.60 ± 0.42	NS
∑ MUFA	20.63 ± 0.90	20.16 ± 1.06	20.28 ± 1.21	NS
22:3n-9	0.31 ± 0.05	0.31 ± 0.08	0.28 ± 0.04	NS
ARA	10.83 ± 0.34	11.04 ± 0.42	11.04 ± 0.33	NS
22:4n-6	3.99 ± 0.34	4.12 ± 0.38	4.20 ± 0.36	NS
22:5n-6	5.90 ± 0.50	5.82 ± 0.70	5.58 ± 0.57	NS
∑ n-6	23.32 ± 0.73	23.62 ± 0.50	23.53 ± 0.80	NS
20:5n-3	0.12 ± 0.03	0.10 ± 0.04	0.12 ± 0.04	NS
22:5n-3	0.31 ± 0.04	0.31 ± 0.05	0.31 ± 0.04	NS
DHA	10.17 ± 0.71	9.97 ± 0.79	10.24 ± 0.39	NS
∑ n-3	10.67 ± 0.70	10.48 ± 0.78	10.76 ± 0.41	NS
ARA/DHA	1.07 ± 0.08	1.11 ± 0.08	1.08 ± 0.04	NS
∑ 22C HUFA[3]	20.67 ± 0.52	20.54 ± 0.60	20.62 ± 0.64	NS
Retina				
∑ SFA	41.92 ± 0.92	42.65 ± 0.90	42.13 ± 1.19	NS
∑ MUFA	17.64 ± 0.37	17.71 ± 0.52	17.83 ± 0.60	NS
22:3n-9	0.09 0.02	0.08 0.02	0.08 0.03	NS
ARA	10.45 ± 0.42	10.42 ± 0.43	10.63 ± 0.45	NS
22:4n-6	2.51 ± 0.19	2.45 ± 0.16	2.43 ± 0.11	NS
22:5n-6	2.58 0.41	2.41 0.56	2.36 0.41	NS
∑ n-6	19.06 ± 0.85	18.90 ± 0.92	18.88 ± 0.68	NS
20:5n-3	0.46 ± 0.07	0.48 ± 0.09	0.40 ± 0.12	NS
22:5n-3	0.62 ± 0.07	0.64 ± 0.07	0.62 ± 0.06	NS
DHA	19.87 ± 0.65	19.20 ± 0.83	19.75 ± 1.66	NS
∑ n-3	21.02 ± 0.69	20.38 ± 0.85	20.83 ± 1.76	NS
ARA/DHA	0.53 ± 0.02	0.54 ± 0.03	0.54 ± 0.05	NS
∑ 22C HUFA	25.58 ± 0.90	24.70 ± 1.06	25.16 ± 1.58	NS

[1]Values represent means ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]SFA, saturated FA; MUFA, monounsaturated FA.

[3]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

FA; P = 0.009). Experimental diets equally supported DHA accretion in the brain, retina and heart, while in the liver, DHA levels were 7% higher in a1 pigs (8.23 ± 0.38) compared with the Control (7.70 ± 0.47% FA) and a2 (7.62 ± 0.62% FA) groups (P = 0.046). No other statistically significant differences in tissue FA accretion were observed among the dietary treatments.

Mean ARA levels in the RBC fraction were similar among all dietary treatment groups at every time point examined (days 3, 7, 14 and 21; Table 3.6). RBC DHA values were similar among dietary treatment groups on days 3 and 21 of age, averaging 1.74 ± 0.32% and 2.57 ± 0.27% of total FA, respectively, but were significantly affected by diet on days 7 and 14 (Figure 3.3). On day 7 of age, RBC DHA levels were significantly higher in the a1 pigs (1.86 ± 0.19% FA) compared with a2 group (1.69 ± 0.26% FA), while Control pigs had intermediary levels (1.81 ± 0.27% FA; P = 0.03). On day 14 of age, RBC DHA levels were the highest in the a1 pigs (2.85 ± 0.31% FA) and were similar between the Control (2.59 ± 0.37% FA) and a2 (2.49 ± 0.14% FA) groups (P = 0.02).

Mean RBC ARA, 20:5n-3, 22:5n-3, 22:4n-6 and 22:5n-6 were similar for all dietary treatment groups at every time point examined. Because of this, treatment groups were pooled and linear regression analysis was performed to determine the temporal pattern of LCPUFA content in RBC. Over the course of the experimental period, mean RBC ARA levels decreased from 6.98 ± 0.60% of total FA on day 3 of age to 4.62 ± 0.32% FA on day 21 of age (P < 0.0001; Figure 3.3). Oneway analysis of RBC ARA content by day of age confirmed this temporal decline, with each incremental increase in age corresponding to a significant decrease in RBC ARA (P < 0.0001). Mean RBC

Table 3.6. LCPUFA composition of RBC collected from piglets on days 3, 7, 14 and 21 of life[1]

Diet	Control	a1	a2	*P*
Day 3		*FA (% total FA)*		
ARA	7.06 ± 0.65	7.04 ± 0.67	6.84 ± 0.52	NS
22:4n-6	0.97 ± 0.17	0.99 ± 0.14	1.00 ± 0.20	NS
22:5n-6	1.96 ± 0.30	1.92 ± 0.12	1.84 ± 0.26	NS
20:5n-3	0.16 ± 0.08	0.17 ± 0.11	0.14 ± 0.03	NS
22:5n-3	0.49 ± 0.08	0.53 ± 0.19	0.49 ± 0.14	NS
DHA	1.78 ± 0.37	1.70 ± 0.25	1.73 ± 0.36	NS
Day 7				
ARA	5.94 ± 0.42	6.09 ± 0.47	5.76 ± 0.27	NS
22:4n-6	0.72 ± 0.07	0.77 ± 0.08	0.69 ± 0.08	NS
22:5n-6	1.09 ± 0.25	1.23 ± 0.10	1.03 ± 0.18	NS
20:5n-3	0.16 ± 0.06	0.16 ± 0.06	0.14 ± 0.03	NS
22:5n-3	0.50 ± 0.07	0.52 ± 0.08	0.48 ± 0.07	NS
DHA	1.81 ± 0.27^{ab}	1.86 ± 0.19^{a}	1.69 ± 0.26^{b}	0.03
Day 14				
ARA	5.04 ± 0.48	5.32 ± 0.37	4.89 ± 0.22	NS
22:4n-6	0.60 ± 0.08	0.61 ± 0.06	0.57 ± 0.03	NS
22:5n-6	0.50 ± 0.05	0.54 ± 0.12	0.46 ± 0.08	NS
20:5n-3	0.22 ± 0.06	0.22 ± 0.06	0.21 ± 0.03	NS
22:5n-3	0.50 ± 0.06	0.55 ± 0.05	0.51 ± 0.04	NS
DHA	2.59 ± 0.37^{b}	2.85 ± 0.31^{a}	2.49 ± 0.14^{b}	0.02
Day 21				
ARA	4.71 ± 0.39	4.55 ± 0.49	4.59 ± 0.23	NS
22:4n-6	0.48 ± 0.06	0.50 ± 0.07	0.50 ± 0.07	NS
22:5n-6	0.24 ± 0.05	0.25 ± 0.06	0.24 ± 0.05	NS
20:5n-3	0.20 ± 0.09	0.18 ± 0.08	0.15 ± 0.09	NS
22:5n-3	0.48 ± 0.05	0.47 ± 0.05	0.48 ± 0.04	NS
DHA	2.61 ± 0.29	2.59 ± 0.26	2.52 ± 0.27	NS

[1]Values represent means ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

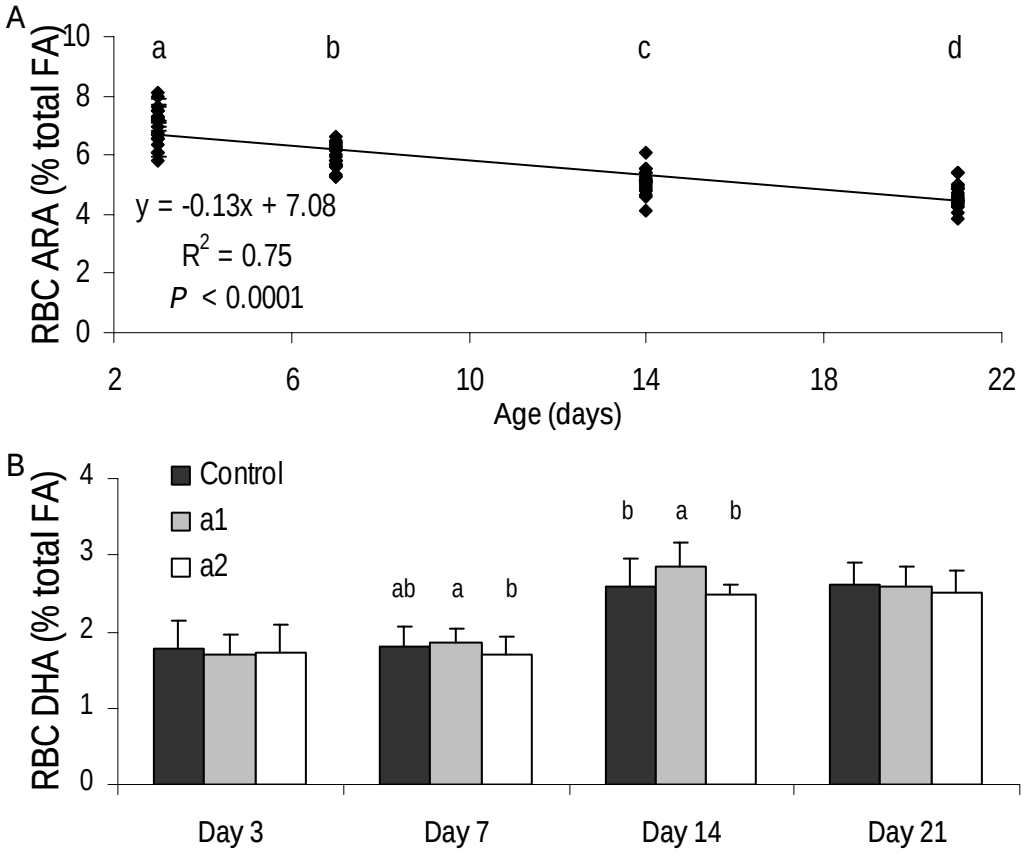

Figure 3.3. Temporal pattern of ARA (A) and DHA (B) content in RBC collected from piglets on days 3, 7, 14 and 21 of life. (A) represents linear regression analysis of RBC ARA content by age; each point corresponds to one pig. In (B), values represent means ± SD, n = 8 per diet. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

22:4n-6 (P < 0.0001) and 22:5n-6 (P < 0.0001) also decreased during the study, while 20:5n-3 and 22:5n-3 levels remained constant.

Discussion

Preclinical studies with animals provide an invaluable tool for determining the safety of novel food additives for use in the diets of humans. In the present study, we evaluated two proprietary ARA oils for potential use in infant formula for toxicological effects and nutritional bioequivalence compared with a commercially available ARA source. Domestic piglets were used because of their rapid rates of growth and similarities to humans in terms of brain development and metabolism of lipids and LCPUFA (22,23).

Growth performance is a well-accepted measure of health and vitality in developing infants. In the present study, the two experimental ARA formulas were readily consumed and equally supported growth in the neonatal pigs compared with the Control formula. Body weights were similar among the three dietary treatment groups at every time point and no differences were observed for organ weights upon sacrifice. The 2000 Center for Disease Control growth charts show that human infants typically double their birth weight by 4 – 5 months of life and grow to 4 times their birth weight by 34 – 36 months (24). In comparison, our piglets grew to 4 times their initial body weight by day 22 of age. This rapid rate of growth and concomitant high demand for formula suggests that any trace toxicological constituent of the formulas could have manifested as poorer growth or suboptimal clinical conditions during this short study duration. Rather, pigs in this study appeared healthy and presented with few health ailments. Loose stools in a number of piglets were the greatest concern during the first week of the study. This was

likely caused by stresses from the transition period and adaptation to a new diet and environment (25), as opposed to a specific diet or dietary component.

Mean values for clinical chemistry and hemogram parameters were consistent with previous studies in neonatal pigs (26-28), and very few differences were observed among the three dietary treatment groups. Control pigs showed elevated plasma AST and creatine kinase compared with pigs fed either of the experimental formulas. For AST, a biomarker of tissue inflammation, Control values were within limits previously observed in nursery pigs (26) and weaned growing pigs (29), suggesting that the numerical difference among diets is likely biologically insignificant. Creatine kinase levels were highest in the Control pigs compared with the two experimental ARA groups, although all treatment means were at the low end of the normal range for commercial pigs (30) and were comparable to values previously observed in a similar neonatal pig study (Chapter One). In all pigs, liver sections were determined to be normal following pathohistological examination and showed no indication of gross abnormalities or toxicological changes. These results are consistent with other studies in pigs showing that the provision of an ARA-rich oil in the milk replacer formula up to 5x the level currently used in formula is safe and produces no adverse effect on serum clinical indicators or liver histology (27,31). Thus, results from the present study indicate that the two experimental ARA oils produce no pathohistological abnormalities in the liver and have no adverse effect on clinical chemistry or hemogram parameters in rapidly growing piglets compared with the Control formula containing ARASCO.

The three ARA oils equally supported ARA accretion in the brain, retina and heart. On the other hand, liver ARA levels showed a remarkable

sensitivity to the dietary ARA content with the Control pigs having significantly higher ARA levels than the a1 group. This discrepancy in liver ARA levels is consistent with the a1 diet providing 8% less ARA (0.62% vs. 0.67% total FA) than the Control and a2 diets, a difference that may arise from normal variability across product lots and over time during in the manufacturing process. However, liver DHA levels also appeared responsive to the difference in dietary ARA level, with the a1 pigs having the highest DHA content. Numerous studies have demonstrated a sensitivity of liver LCPUFA to dietary FA composition (32,33)(Chapter Two) that occurs primarily as a result of dietary FA composition but also due to the regulation of LCPUFA biosynthesis by dietary LCPUFA (34,35). Overall, these results demonstrate that the experimental ARA oils are nutritionally bioequivalent to ARASCO and that subtle variations in total formula ARA content may influence liver ARA and DHA levels and endogenous LCPUFA biosynthesis.

Studies with human infants are limited by the information they provide, especially with regard to the ethical limitations and potential risks associated with frequent blood sampling as well as the general unavailability of tissue from healthy individuals (36). This study provided a unique opportunity to examine acute changes in RBC LCPUFA levels that may have been otherwise overlooked in clinical studies with infants. The lack of significant differences among dietary treatment groups permitted pooling of sample means to determine the temporal pattern of RBC LCPUFA in pigs fed 0.64% ARA and 0.32%. Here, we observed significant decreases in RBC ARA, 22:4n-6 and 22:5n-6 and an increase in RBC DHA compared with baseline values. The rapid and dramatic decline in RBC ARA has not been previously documented in neonatal animals and the cause remains unclear. Human infants that

consume breast milk or formula with added LCPUFA show a modest decline in RBC ARA during the first year of life (37). At the same time, the infant brain rapidly accumulates ARA (4), and in Chapter Two, we demonstrated that the growing heart specifically incorporates and concentrates ARA, and that ARA accretion is limited by dietary ARA content. It is probable that the temporal decline in RBC ARA we observed in the present study occurred as a result of rapid tissue ARA accretion. With regard to DHA, we observed a general increase in RBC DHA that appeared to reach plateau by day 14 of age. The low levels of RBC DHA may be explained by the negligible content of DHA in the sow milk previously analyzed from this herd (Chapters One and Two), while the content of RBC DHA on days 14 and 21 are consistent with most (38-41), but not all (42) studies of rapidly growing, neonatal pigs consuming low DHA.

In 2001, ARASCO was determined to be GRAS for use in term infant formula up to 1.88% of total fat and providing ARA up to 0.75% of total fat (12). More recently, SUNTGA40S was determined to be GRAS for preterm and term infants when providing ARA up to 0.40% of total fat (14), and a third ARA-rich oil, RAO is currently pending FDA approval for use in infant formula (15). It is possible that the experimental oils used in this study are SUNTGA40S, evaluated at a higher formula content than is currently approved for use in formula, and RAO which has not been reported in preclinical studies with neonatal pigs. However, we cannot exclude the possibility that the experimental ARA oils are of a novel, proprietary formulation that may or may not be derived from *M. alpina* and for which little safety data is known.

Overall, this study evaluated the safety and bioefficacy of three ARA-rich oils for potential use in commercial infant formula. Oils provided

ARA at 0.64% of total FA, were added with DHASCO to provide 0.34% DHA and resulted in no other major alterations in the formula FA composition. We conclude that the two experimental ARA sources were nutritionally bioequivalent to ARASCO, each equally supporting ARA accretion in the brain, retina and heart, and produced no toxicological effects in neonatal pigs. Results further demonstrate that ARA-rich oils that deliver equivalent levels of ARA equally support the content of ARA in formula when prepared using a common manufacturing process.

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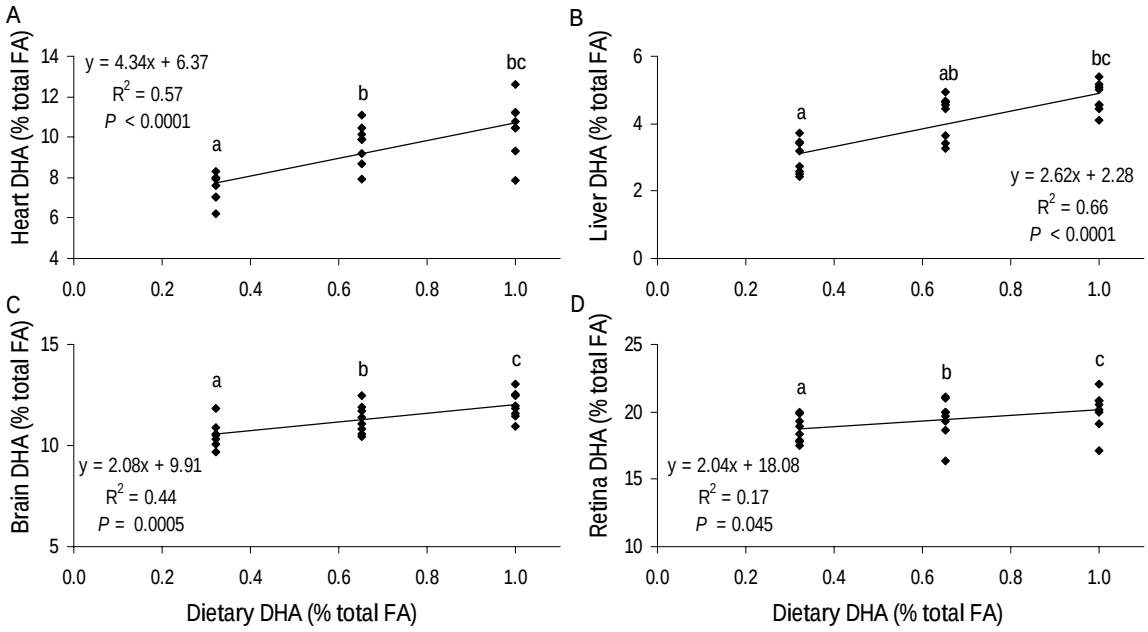
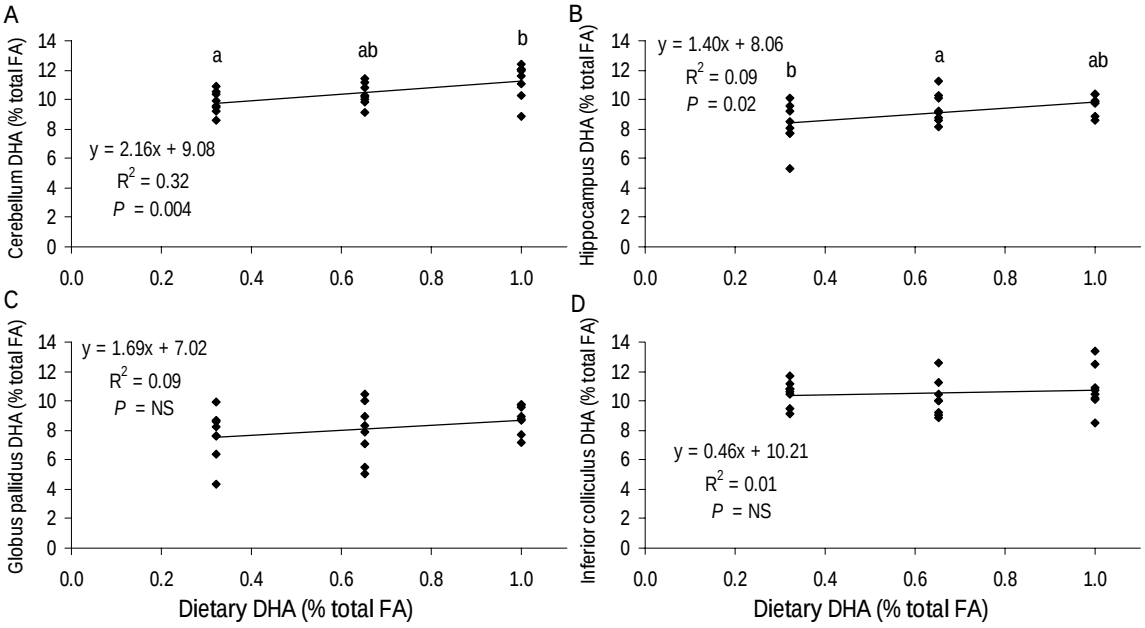
APPENDIX I

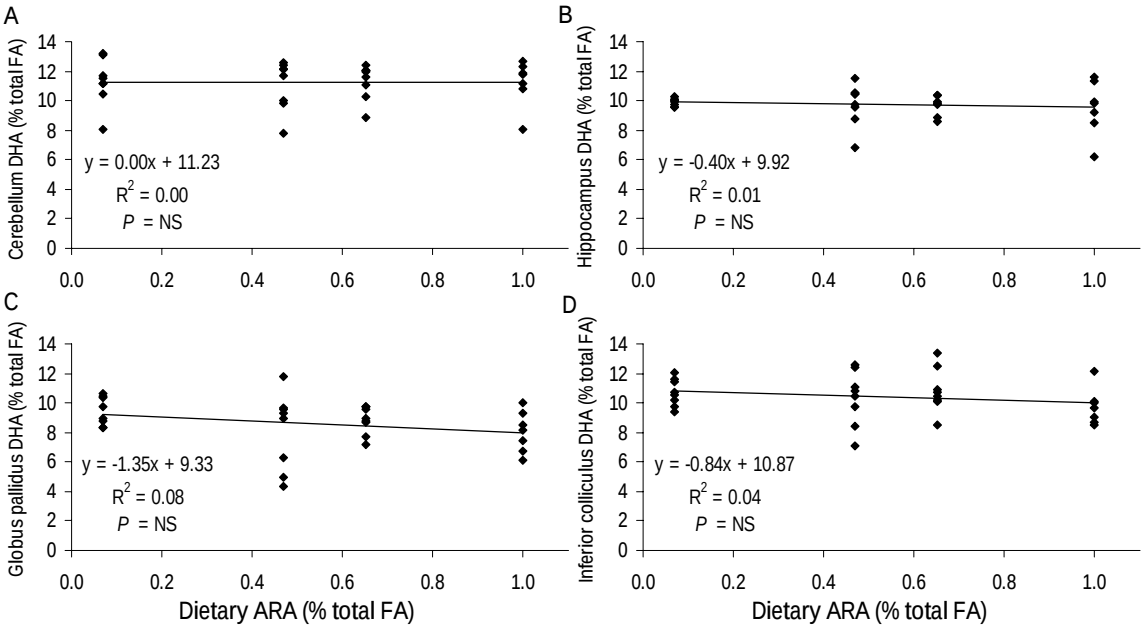
SUPPLEMENTAL FIGURES AND TABLES FOR CHAPTER TWO



Supplemental Figure 1. DHA tissue dose-response to dietary DHA with constant ARA (0.67% total FA). Dietary DHA content for d1, d2 and d3 was 0.32, 0.62 and 1.01% total FA, respectively. Tissue DHA rises with dietary DHA in all tissues presented, (A) Heart, (B) Liver, (C) Brain (Cerebral cortex) and (D) Retina. Each point represents one animal (n = 24 per panel). $P < 0.05$ indicates significant correlation between dietary DHA content and tissue DHA level; NS, not significant. Student's t-test used to determine significance of pairwise comparisons of mean tissue DHA content; means not sharing a common letter are significantly different ($P < 0.05$).



Supplemental Figure 2. Neural tissue DHA dose-response to dietary DHA with constant ARA (0.67% total FA). Dietary DHA content for d1, d2 and d3 was 0.32, 0.62 and 1.01% total FA, respectively. Tissue DHA rises with dietary DHA in all tissues presented, (A) Cerebellum, (B) Hippocampus, (C) Globus pallidus and (D) Inferior colliculus. Mean DHA levels in Hippocampus, Globus pallidus and Inferior colliculus were 17%, 15% and 3% higher in d3 pigs compared with d1 pigs, although these differences were not statistically significant. Each point represents one animal (n = 24 per panel). $P < 0.05$ indicates significant correlation between dietary DHA content and tissue DHA level; NS, not significant. Student's t-test used to determine significance of pairwise comparisons of mean tissue DHA content; means not sharing a common letter are significantly different ($P < 0.05$).



Supplemental Figure 3. Neural tissue DHA dose-response to dietary ARA content with constant DHA (1.0% total FA). Dietary ARA content for a1, a2, a3 and a4 was 0.09, 0.53, 0.69 and 1.06% total FA, respectively. Increasing ARA had no effect on DHA levels in (A) Cerebellum, (B) Hippocampus, (C) Globus pallidus or (D) Inferior colliculus. Each point represents one animal (n = 24 per panel). $P < 0.05$ indicates significant correlation between dietary ARA content and tissue DHA level; NS, not significant. Student's t-test used to determine significance of pairwise comparisons of mean tissue DHA content; means not sharing a common letter are significantly different ($P < 0.05$).

Supplemental Table 1. FA composition of heart from piglets fed varying levels of ARA and DHA from days 3 - 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
Σ SFA	32.81 ± 1.00^{bc}	32.16 ± 0.55^{c}	32.63 ± 0.69^{bc}	32.62 ± 0.62^{bc}	32.64 ± 0.61^{ab}	33.36 ± 0.90^{a}	32.60 ± 0.66^{abc}	0.006
Σ MUFA	16.78 ± 1.05	17.74 ± 0.99	17.35 ± 1.05	17.50 ± 0.50	17.10 ± 0.88	17.59 ± 0.94	17.17 ± 0.75	NS
ARA	14.76 ± 1.07^{d}	18.57 ± 0.67^{c}	19.41 ± 1.00^{b}	20.90 ± 0.93^{a}	19.98 ± 1.38^{ab}	19.09 ± 1.88^{bc}	20.26 ± 1.20^{ab}	<.0001
22:4n-6	0.63 ± 0.09^{e}	0.71 ± 0.09^{e}	0.71 ± 0.06^{de}	0.82 ± 0.09^{cd}	0.88 ± 0.13^{bc}	0.96 ± 0.12^{b}	1.33 ± 0.13^{a}	<.0001
22:5n-6	0.00 ± 0.01^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.02 ± 0.03^{b}	0.08 ± 0.01^{a}	<.0001
Σ n-6	43.29 ± 1.09^{bc}	42.95 ± 0.55^{c}	43.45 ± 0.96^{bc}	43.19 ± 0.77^{bc}	44.12 ± 0.88^{b}	44.06 ± 1.20^{b}	46.85 ± 0.97^{a}	<.0001
20:5n-3	0.75 ± 0.09^{a}	0.54 ± 0.09^{b}	0.49 ± 0.04^{b}	0.41 ± 0.04^{c}	0.42 ± 0.04^{c}	0.32 ± 0.03^{d}	0.28 ± 0.06^{d}	<.0001
22:5n-3	0.59 ± 0.07^{d}	0.59 ± 0.08^{d}	0.57 ± 0.04^{d}	0.57 ± 0.04^{d}	0.71 ± 0.07^{c}	0.83 ± 0.13^{b}	1.24 ± 0.07^{a}	<.0001
DHA	4.81 ± 0.24^{b}	5.20 ± 0.49^{a}	4.78 ± 0.43^{b}	5.01 ± 0.25^{ab}	4.20 ± 0.64^{c}	3.00 ± 0.50^{d}	0.75 ± 0.08^{e}	<.0001
Σ n-3	6.58 ± 0.21^{ab}	6.73 ± 0.57^{a}	6.24 ± 0.42^{b}	6.37 ± 0.31^{ab}	5.71 ± 0.67^{c}	4.57 ± 0.51^{d}	2.48 ± 0.11^{e}	<.0001
ARA/DHA	3.07 ± 0.19^{e}	3.60 ± 0.39^{de}	4.08 ± 0.34^{cd}	4.18 ± 0.22^{cd}	4.83 ± 0.58^{c}	6.46 ± 0.78^{b}	27.26 ± 3.17^{a}	<.0001
∑ 22C HUFA[4]	6.04 ± 0.25^{bc}	6.50 ± 0.51^{a}	6.06 ± 0.4^{abc}	6.40 ± 0.23^{ab}	5.78 ± 0.72^{c}	4.81 ± 0.59^{d}	3.40 ± 0.15^{e}	<.0001

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 2. FA composition of liver from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1			a2			a3-d3			a4			d2			d1			MR[2]			
ARA/DHA[3]	0.09/1.00			0.53/1.02			0.69/1.01			1.06/1.04			0.67/0.62			0.66/0.33			0.74/0.01			*P*
	FA (% total FA)																					
∑ SFA	42.89	±	1.78	42.11	±	0.44	42.88	±	1.88	42.47	±	2.23	42.29	±	0.77	42.71	±	1.62	39.97	±	1.50	NS
∑ MUFA	13.40	±	1.08^{b}	12.77	±	1.11^{b}	12.75	±	0.83^{b}	13.17	±	0.99^{b}	13.12	±	0.77^{b}	13.21	±	0.68^{b}	19.51	±	3.80^{a}	<.0001
20:4 (n-6)	13.92	±	1.49^{d}	16.14	±	0.87^{c}	16.12	±	1.10^{bc}	16.88	±	1.50^{b}	17.36	±	0.63^{b}	17.87	±	1.46^{a}	17.00	±	1.36^{abc}	<.0001
22:4 (n-6)	1.08	±	0.29^{d}	1.15	±	0.22^{cd}	1.27	±	0.22^{c}	1.36	±	0.22^{bc}	1.28	±	0.20^{bc}	1.50	±	0.10^{b}	2.27	±	0.35^{a}	<.0001
22:5 (n-6)	0.03	±	0.06^{d}	0.06	±	0.05^{d}	0.04	±	0.04^{d}	0.06	±	0.07^{d}	0.20	±	0.09^{c}	0.37	±	0.11^{b}	1.89	±	0.53^{a}	<.0001
∑ (n-6)	30.25	±	1.10^{d}	31.34	±	0.58^{cd}	31.57	±	1.01^{bc}	31.70	±	1.34^{bc}	32.58	±	0.64^{b}	34.05	±	1.39^{a}	35.16	±	1.66^{a}	<.0001
20:5 (n-3)	0.61	±	0.14^{a}	0.42	±	0.15^{bc}	0.45	±	0.10^{b}	0.36	±	0.11^{cd}	0.34	±	0.05^{cd}	0.31	±	0.04^{d}	0.18	±	0.06^{e}	<.0001
22:5 (n-3)	0.94	±	0.11^{c}	0.91	±	0.09^{c}	0.94	±	0.12^{c}	0.94	±	0.14^{c}	1.01	±	0.07^{c}	1.11	±	0.16^{b}	1.35	±	0.20^{a}	<.0001
22:6 (n-3)	10.77	±	1.77^{a}	11.43	±	1.22^{a}	10.49	±	1.41^{a}	10.45	±	1.79^{a}	9.65	±	1.01^{b}	7.52	±	0.69^{c}	2.37	±	0.30^{d}	<.0001
∑ (n-3)	12.67	±	1.79^{a}	13.08	±	1.15^{a}	12.22	±	1.57^{a}	12.11	±	1.90^{a}	11.34	±	1.10^{b}	9.29	±	0.76^{c}	4.13	±	0.49^{d}	<.0001
ARA/DHA	1.31	±	0.17^{f}	1.43	±	0.19^{ef}	1.55	±	0.16^{de}	1.65	±	0.24^{cd}	1.82	±	0.21^{c}	2.39	±	0.20^{b}	7.21	±	0.42^{a}	<.0001
∑ 22C HUFA[4]	12.82	±	1.86^{a}	13.55	±	1.26^{a}	12.74	±	1.44^{ab}	12.81	±	1.90^{ab}	12.14	±	1.21^{b}	10.51	±	0.81^{c}	7.88	±	1.22^{d}	<.0001

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 3. FA composition of cerebral cortex from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
∑ SFA	45.67 ± 0.53^{ab}	45.77 ± 0.81^{ab}	46.10 ± 0.90^{a}	46.04 ± 1.10^{a}	45.27 ± 0.74^{bc}	45.93 ± 0.79^{a}	44.14 ± 0.64^{c}	0.005
∑ MUFA	18.55 ± 0.53^{bc}	18.87 ± 0.75^{bc}	18.34 ± 0.67^{c}	18.27 ± 0.77^{c}	18.70 ± 0.44^{bc}	18.95 ± 0.76^{b}	19.97 ± 0.93^{a}	0.006
22:3n-9	0.23 ± 0.06^{b}	0.21 ± 0.03^{b}	0.21 ± 0.02b	0.22 ± 0.06^{b}	0.23 ± 0.04^{b}	0.19 ± 0.03^{b}	0.31 ± 0.00^{a}	0.009
20:4n-6	11.95 ± 0.29	11.82 ± 0.60	11.80 ± 0.35	12.04 ± 0.63	11.93 ± 0.40	11.77 ± 0.68	12.33 ± 0.00	NS
22:4n-6	4.03 ± 0.52	4.06 ± 0.16	4.09 ± 0.35	4.21 ± 0.60	4.21 ± 0.32	4.19 ± 0.26	4.81 ± 0.30	NS
22:5n-6	4.49 ± 0.40^{c}	4.44 ± 0.47^{c}	4.68 ± 0.29^{c}	4.61 ± 0.56^{c}	5.52 ± 0.64^{b}	5.49 ± 0.25^{b}	8.26 ± 0.18^{a}	<.0001
∑ n-6	22.58 ± 0.77^{d}	22.36 ± 1.03^{d}	22.54 ± 0.92^{d}	22.78 ± 1.40^{cd}	23.65 ± 0.85^{bc}	23.56 ± 0.80^{b}	27.62 ± 0.50^{a}	<.0001
20:5n-3	0.06 ± 0.01^{b}	0.06 ± 0.02^{b}	0.06 ± 0.02^{b}	0.07 ± 0.01^{b}	0.05 ± 0.02^{b}	0.06 ± 0.02^{b}	0.11 ± 0.03^{a}	0.005
22:5n-3	0.20 ± 0.02^{c}	0.19 ± 0.02^{c}	0.19 ± 0.04^{c}	0.19 ± 0.03^{c}	0.20 ± 0.02^{c}	0.25 ± 0.05^{b}	0.32 ± 0.03^{a}	<.0001
22:6n-3	12.10 ± 0.83^{a}	11.91 ± 0.74^{ab}	11.98 ± 0.69^{ab}	11.90 ± 0.69^{ab}	11.30 ± 0.70^{b}	10.56 ± 0.67^{c}	6.88 ± 0.55^{d}	<.0001
∑ n-3	12.36 ± 0.84^{a}	12.16 ± 0.76^{ab}	12.23 ± 0.71^{ab}	12.16 ± 0.69^{ab}	11.55 ± 0.72^{b}	10.87 ± 0.67^{c}	7.31 ± 0.55^{d}	<.0001
ARA/DHA	0.99 ± 0.07^{c}	1.00 ± 0.09^{c}	0.99 ± 0.05^{c}	1.02 ± 0.10^{bc}	1.06 ± 0.09^{bc}	1.12 ± 0.11^{b}	1.80 ± 0.17^{a}	<.0001
∑ 22C HUFA[4]	20.82 ± 0.81	20.60 ± 0.61	20.94 ± 1.01	20.91 ± 0.99	21.23 ± 0.88	20.49 ± 0.79	20.27 ± 0.72	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 4. FA composition of retina from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
Σ SFA	44.70 ± 0.83	44.39 ± 0.51	44.89 ± 0.97	44.63 ± 0.91	44.40 ± 1.13	44.57 ± 0.69	43.61 ± 0.32	NS
Σ MUFA	18.40 ± 0.59	18.38 ± 0.37	18.48 ± 0.57	18.24 ± 0.65	18.63 ± 0.71	18.72 ± 0.43	19.50 ± 0.70	NS
22:3n-9	0.10 ± 0.03	0.11 ± 0.02	0.10 ± 0.02	0.10 ± 0.10	0.13 ± 0.02	0.11 ± 0.03	0.12 ± 0.02	NS
20:4n-6	9.48 ± 0.23^{c}	9.70 ± 0.61^{bc}	9.66 ± 0.32^{bc}	9.81 ± 0.31^{b}	9.96 ± 0.37^{b}	9.95 ± 0.25^{b}	10.74 ± 0.50^{a}	0.0004
22:4n-6	1.66 ± 0.15^{e}	1.72 ± 0.12^{de}	1.77 ± 0.12^{d}	1.81 ± 0.17^{d}	1.98 ± 0.19^{c}	2.13 ± 0.12^{b}	2.98 ± 0.17^{a}	<.0001
22:5n-6	1.48 ± 0.30^{d}	1.48 ± 0.32^{d}	1.45 ± 0.16^{d}	1.55 ± 0.30^{d}	2.03 ± 0.64^{c}	2.29 ± 0.26^{b}	5.24 ± 0.39^{a}	<.0001
Σ n-6	15.31 ± 0.54^{d}	15.42 ± 0.89^{d}	15.41 ± 0.47^{d}	15.57 ± 0.70^{d}	16.37 ± 0.91^{c}	16.98 ± 0.24^{b}	21.65 ± 0.96^{a}	<.0001
20:5n-3	0.18 ± 0.05^{ab}	0.15 ± 0.04^{b}	0.18 ± 0.05^{a}	0.13 ± 0.02^{c}	0.10 ± 0.04^{c}	0.08 ± 0.02^{d}	0.02 ± 0.01^{e}	<.0001
22:5n-3	0.64 ± 0.04^{a}	0.60 ± 0.05^{b}	0.63 ± 0.06^{ab}	0.60 ± 0.03^{b}	0.55 ± 0.03^{c}	0.60 ± 0.05^{b}	0.60 ± 0.05^{abc}	0.0001
22:6n-3	20.42 ± 1.20^{ab}	20.70 ± 0.54^{a}	20.07 ± 1.47^{ab}	20.49 ± 1.31^{ab}	19.50 ± 1.50^{bc}	18.68 ± 0.96^{c}	14.16 ± 1.37^{d}	<.0001
Σ n-3	21.24 ± 1.22^{ab}	21.44 ± 0.57^{a}	20.89 ± 1.48^{ab}	21.22 ± 1.34^{ab}	20.15 ± 1.55^{bc}	19.36 ± 0.99^{c}	14.78 ± 1.36^{d}	<.0001
ARA/DHA	0.47 ± 0.03^{c}	0.47 ± 0.04^{c}	0.48 ± 0.04^{c}	0.48 ± 0.04^{bc}	0.51 ± 0.04^{bc}	0.53 ± 0.03^{b}	0.77 ± 0.11^{a}	<.0001
∑ 22C HUFA[4]	24.29 ± 1.34	24.61 ± 0.49	24.03 ± 1.49	24.55 ± 1.48	24.20 ± 1.31	23.81 ± 0.90	23.10 ± 0.98	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 5. FA composition of cerebellum from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
Σ SFA	41.83 ± 1.91	42.29 ± 2.00	42.72 ± 1.43	42.12 ± 1.93	42.41 ± 1.66	42.40 ± 2.31	41.57 ± 0.85	NS
Σ MUFA	27.22 ± 0.99	26.93 ± 1.50	25.95 ± 1.04	27.07 ± 1.30	26.35 ± 1.60	25.98 ± 2.60	27.20 ± 1.43	NS
22:3n-9	0.42 ± 0.07^{abc}	0.38 ± 0.05^{c}	0.37 ± 0.04^{c}	0.37 ± 0.04^{c}	0.44 ± 0.04^{ab}	0.38 ± 0.07^{bc}	0.48 ± 0.06^{a}	0.009
20:4n-6	10.40 ± 0.30^{c}	10.76 ± 0.26^{bc}	11.03 ± 0.31^{ab}	10.44 ± 0.44^{c}	10.95 ± 0.62^{ab}	10.78 ± 0.43^{bc}	11.48 ± 0.73^{a}	0.007
22:4n-6	3.29 ± 0.41^{d}	3.23 ± 0.38^{d}	3.39 ± 0.32^{cd}	3.43 ± 0.32^{cd}	3.55 ± 0.19^{c}	3.97 ± 0.25^{b}	4.66 ± 0.31^{a}	<.0001
22:5n-6	2.12 ± 0.26^{cd}	2.19 ± 0.34^{d}	2.18 ± 0.19^{cd}	2.17 ± 0.26^{d}	2.70 ± 0.37^{bc}	3.34 ± 1.09^{b}	4.82 ± 0.23^{a}	<.0001
Σ n-6	17.98 ± 1.09^{d}	18.15 ± 1.17^{d}	18.47 ± 0.84^{cd}	18.07 ± 0.64^{d}	19.22 ± 0.65^{c}	20.27 ± 1.12^{b}	23.26 ± 0.92^{a}	<.0001
20:5n-3	0.21 ± 0.03	0.22 ± 0.04	0.20 ± 0.05	0.22 ± 0.05	0.20 ± 0.06	0.20 ± 0.07	0.21 ± 0.05	NS
22:5n-3	0.35 ± 0.06^{bc}	0.31 ± 0.05^{cd}	0.32 ± 0.05^{cd}	0.32 ± 0.05^{d}	0.31 ± 0.02^{cd}	0.36 ± 0.05^{b}	0.47 ± 0.04^{a}	<.0001
22:6n-3	11.29 ± 1.61^{a}	11.08 ± 1.68^{a}	11.29 ± 1.19^{a}	11.19 ± 1.44^{a}	10.37 ± 0.75^{ab}	9.83 ± 0.76^{b}	6.10 ± 0.28^{c}	<.0001
Σ n-3	11.85 ± 1.67^{a}	11.61 ± 1.71^{a}	11.82 ± 1.21^{a}	11.73 ± 1.47^{a}	10.88 ± 0.79^{ab}	10.39 ± 0.81^{b}	6.77 ± 0.28^{c}	<.0001
ARA/DHA	0.91 ± 0.09^{d}	0.96 ± 0.11^{d}	0.97 ± 0.07^{cd}	0.95 ± 0.11^{cd}	1.06 ± 0.12^{bc}	1.10 ± 0.09^{b}	1.89 ± 0.20^{a}	<.0001
∑ 22C HUFA[4]	17.47 ± 1.79	17.19 ± 2.23	17.56 ± 1.51	17.47 ± 1.54	17.36 ± 0.70	17.88 ± 1.37	16.53 ± 0.30	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 6. FA composition of hippocampus from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
∑ SFA	41.80 ± 1.32^{abc}	42.33 ± 1.12^{ab}	41.48 ± 1.62^{c}	41.13 ± 1.76^{bc}	42.51 ± 0.94^{ab}	41.53 ± 1.34^{bc}	43.67 ± 0.520^{a}	0.046
∑ MUFA	26.65 ± 3.10	25.62 ± 3.24	27.02 ± 4.01	27.44 ± 5.57	24.75 ± 2.42	26.92 ± 3.89	23.67 ± 1.48	NS
22:3n-9	0.48 ± 0.08	0.46 ± 0.12	0.49 ± 0.12	0.45 ± 0.09	0.46 ± 0.10	0.52 ± 0.15	0.47 ± 0.12	NS
20:4n-6	10.40 ± 0.89	10.78 ± 0.93	10.57 ± 1.09	10.61 ± 1.57	11.10 ± 0.97	10.85 ± 1.23	11.77 ± 0.96	NS
22:4n-6	4.57 ± 0.36	4.68 ± 0.43	4.63 ± 0.57	4.72 ± 0.63	4.78 ± 0.44	5.03 ± 0.51	5.37 ± 0.43	NS
22:5n-6	2.89 ± 0.22^{c}	2.96 ± 0.39^{c}	3.06 ± 0.39^{cd}	2.68 ± 0.67^{d}	3.71 ± 0.40^{b}	3.31 ± 0.53^{bc}	5.58 ± 0.60^{a}	<.0001
∑ n-6	20.27 ± 1.21^{c}	20.62 ± 1.26^{bc}	20.46 ± 1.85^{c}	20.09 ± 2.66^{bc}	21.64 ± 1.40^{b}	21.47 ± 1.94^{b}	24.83 ± 0.99^{a}	0.0001
20:5n-3	0.30 ± 0.14	0.26 ± 0.11	0.32 ± 0.15	0.32 ± 0.21	0.25 ± 0.10	0.29 ± 0.11	0.21 ± 0.05	NS
22:5n-3	0.24 ± 0.03^{ab}	0.22 ± 0.05^{bc}	0.22 ± 0.02^{bc}	0.20 ± 0.05^{bc}	0.19 ± 0.02^{c}	0.24 ± 0.04^{ab}	0.27 ± 0.04^{a}	0.009
22:6n-3	9.89 ± 0.26^{a}	9.74 ± 1.43^{a}	9.66 ± 0.68^{ab}	9.63 ± 1.73^{a}	9.44 ± 1.03^{a}	8.27 ± 1.48^{b}	6.12 ± 0.68^{c}	0.0002
∑ n-3	9.74 ± 1.15^{ab}	9.96 ± 1.45^{ab}	9.45 ± 1.36^{bc}	9.83 ± 1.78^{a}	9.64 ± 1.05^{ab}	8.50 ± 1.50^{c}	6.39 ± 0.70^{d}	<.0001
ARA/DHA	1.10 ± 0.09^{c}	1.12 ± 0.13^{c}	1.16 ± 0.18^{c}	1.11 ± 0.09^{c}	1.18 ± 0.07^{c}	1.33 ± 0.14^{b}	1.93 ± 0.18^{a}	<.0001
∑ 22C HUFA[4]	17.20 ± 1.41	17.60 ± 1.87	17.13 ± 1.99	17.23 ± 2.89	18.12 ± 1.44	16.84 ± 2.23	17.34 ± 0.64	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 7. FA composition of globus pallidus from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
∑ SFA	42.71 ± 1.41	41.00 ± 2.51	42.24 ± 1.05	41.98 ± 1.42	41.56 ± 1.54	41.65 ± 2.00	42.67 ± 1.93	NS
∑ MUFA	27.27 ± 2.77	31.99 ± 6.70	28.36 ± 2.42	29.79 ± 3.98	30.05 ± 4.63	29.86 ± 4.61	26.74 ± 3.75	NS
22:3n-9	0.42 ± 0.08	0.47 ± 0.11	0.47 ± 0.06	0.50 ± 0.10	0.50 ± 0.09	0.48 ± 0.11	0.50 ± 0.09	NS
20:4n-6	10.33 ± 0.68	9.33 ± 1.28	10.07 ± 0.44	9.92 ± 0.76	10.02 ± 0.88	10.09 ± 0.96	10.73 ± 0.82	NS
22:4n-6	3.81 ± 0.32^{bc}	3.59 ± 0.73^{b}	4.25 ± 0.51^{ab}	3.98 ± 0.87^{bc}	3.72 ± 0.37^{bc}	4.04 ± 0.58^{bc}	4.78 ± 0.65^{a}	0.02
22:5n-6	2.93 ± 0.37^{b}	2.24 ± 0.77^{c}	2.81 ± 0.40^{bc}	2.64 ± 0.58^{bc}	3.16 ± 0.73^{b}	3.04 ± 0.78^{b}	5.40 ± 0.92^{a}	<.0001
∑ n-6	18.93 ± 1.02^{b}	17.00 ± 2.41^{c}	18.82 ± 1.15^{bc}	18.18 ± 1.93^{bc}	18.59 ± 1.65^{bc}	18.99 ± 1.88^{b}	22.72 ± 2.22^{a}	0.0002
20:5n-3	0.35 ± 0.11	0.54 ± 0.31	0.39 ± 0.15	0.45 ± 0.17	0.48 ± 0.20	0.44 ± 0.19	0.33 ± 0.15	NS
22:5n-3	0.15 ± 0.12	0.12 ± 0.04	0.13 ± 0.03	0.13 ± 0.02	0.11 ± 0.04	0.14 ± 0.06	0.19 ± 0.06	NS
22:6n-3	9.45 ± 0.97^{a}	8.09 ± 2.61^{ab}	8.80 ± 0.94^{ab}	8.18 ± 1.36^{ab}	7.91 ± 1.95^{b}	7.66 ± 1.68^{bc}	6.08 ± 0.35^{c}	0.01
∑ n-3	9.96 ± 0.89^{a}	8.75 ± 2.34^{ab}	9.33 ± 0.88^{ab}	8.76 ± 1.23^{ab}	8.50 ± 1.79^{b}	8.25 ± 1.57^{b}	6.60 ± 0.29^{c}	0.005
ARA/DHA	1.10 ± 0.07^{d}	1.24 ± 0.30^{bcd}	1.15 ± 0.11^{cd}	1.23 ± 0.15^{bcd}	1.32 ± 0.26^{bc}	1.37 ± 0.30^{b}	1.77 ± 0.15^{a}	<.0001
∑ 22C HUFA[4]	16.74 ± 1.12	14.49 ± 3.72	16.44 ± 1.37	15.41 ± 2.20	15.37 ± 2.68	15.34 ± 2.50	16.92 ± 1.43	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

Supplemental Table 8. FA composition of inferior colliculus from piglets fed varying levels of ARA and DHA from days 3 – 28 of age[1]

Diet	a1	a2	a3-d3	a4	d2	d1	MR[2]	
ARA/DHA[3]	0.09/1.00	0.53/1.02	0.69/1.01	1.06/1.04	0.67/0.62	0.66/0.33	0.74/0.01	*P*
				FA (% total FA)				
∑ SFA	40.88 ± 1.24	41.85 ± 1.15	41.59 ± 1.62	40.66 ± 1.38	41.45 ± 1.16	41.52 ± 1.28	40.94 ± 1.87	NS
∑ MUFA	30.94 ± 2.12	30.92 ± 3.15	30.08 ± 3.70	32.32 ± 2.74	30.81 ± 1.93	29.83 ± 2.49	31.02 ± 4.44	NS
22:3n-9	0.33 ± 0.04^{bc}	0.30 ± 0.04^{c}	0.29 ± 0.06^{c}	0.37 ± 0.07^{b}	0.32 ± 0.07^{bc}	0.29 ± 0.04^{c}	0.48 ± 0.10^{a}	<.0001
20:4n-6	8.58 ± 0.35	8.55 ± 0.75	8.85 ± 0.84	8.61 ± 0.52	8.63 ± 0.50	8.97 ± 0.73	9.45 ± 1.25	NS
22:4n-6	3.54 ± 0.21^{b}	3.36 ± 0.19^{b}	3.55 ± 0.31^{b}	3.56 ± 0.29^{b}	3.42 ± 0.30^{b}	3.70 ± 0.35^{b}	4.68 ± 1.16^{a}	<.0001
22:5n-6	1.58 ± 0.49^{bc}	1.50 ± 0.35^{c}	1.60 ± 0.45^{bc}	1.62 ± 0.52^{bc}	1.93 ± 0.53^{b}	1.91 ± 0.32^{b}	3.76 ± 0.45^{a}	<.0001
∑ n-6	15.97 ± 0.56^{b}	15.53 ± 1.04^{b}	16.12 ± 1.24^{b}	15.86 ± 0.93^{b}	16.14 ± 1.03^{b}	16.77 ± 1.13^{b}	19.93 ± 2.37^{a}	<.0001
20:5n-3	0.39 ± 0.07	0.35 ± 0.12	0.34 ± 0.11	0.43 ± 0.09	0.36 ± 0.05	0.33 ± 0.07	0.39 ± 0.14	NS
22:5n-3	0.19 ± 0.02^{ab}	0.18 ± 0.04^{abc}	0.19 ± 0.04^{ab}	0.15 ± 0.02^{c}	0.16 ± 0.03^{bc}	0.18 ± 0.03^{bc}	0.21 ± 0.06^{a}	0.02
22:6n-3	10.71 ± 0.94^{a}	10.32 ± 1.88^{a}	10.84 ± 1.50^{a}	9.58 ± 1.21^{a}	10.18 ± 1.27^{a}	10.53 ± 0.85^{a}	6.27 ± 1.14^{b}	<.0001
∑ n-3	11.29 ± 0.92^{a}	10.85 ± 1.83^{a}	11.37 ± 1.46^{a}	10.17 ± 1.15^{a}	10.70 ± 1.25^{a}	11.04 ± 0.82^{a}	6.87 ± 1.07^{b}	<.0001
ARA/DHA	0.81 ± 0.06^{b}	0.84 ± 0.11^{b}	0.82 ± 0.08^{b}	0.91 ± 0.10^{b}	0.86 ± 0.09^{b}	0.85 ± 0.06^{b}	1.55 ± 0.32^{a}	0.0001
∑ 22C HUFA[4]	16.35 ± 1.10	15.66 ± 2.18	16.46 ± 2.03	15.28 ± 1.60	16.02 ± 1.46	16.61 ± 1.23	15.40 ± 1.74	NS

[1]Values represent mean ± SD, n = 8 per diet. $P < 0.05$ indicates that all means are not equal; NS, not significant. Pairwise comparisons determined using Student's t-test; means not sharing a common superscript are significantly different ($P < 0.05$).

[2]ARA/DHA, dietary content of ARA/DHA (% FA/FA).

[3]SFA, saturated FA, MUFA, monounsaturated FA.

[4]∑ 22C HUFA = ∑ 22:3n-9, 22:4n-6, 22:5n-6, 22:5n-3, DHA.

APPENDIX II

ONE-STEP HYDROLYSIS, EXTRACTION AND METHYLATION PROCEDURE FOR THE PREPARATION OF FATTY ACID METHYL ESTERS FROM VERTEBRATE SOFT TISSUE

This procedure is adapted from the One-step procedure of Garces and Mancha (1993).

Reagents:

Aqueous Reagent:

Methanol

2,2-Dimethoxypropane

Concentrated sulfuric acid

(85:11:4 by volume)

Organic Reagent:

Heptane

Toluene

(63:37 by volume)

Heptane

Saturated sodium chloride

Di-17:0 phosphatidylcholine, 5.65 ug/ul concentration for internal standard

Protocol:

1. Weigh out sample (~ 50mg)[1] in a 16 x 125 mm test tube.

2. Add internal standard, total amount based on sample type.

3. Add 2 mL heptane.

4. Add 1.4 mL of the aqueous reagent and 1.6 mL of the organic reagent with a glass pipette.

5. Seal cap inside and out with Teflon tape and vortex for 1 min.

6. Place tubes in a heating block at 80°C for two hours.

7. Vortex tubes until they come to room temperature (~10 min).

8. Add 2 mL of saturated sodium chloride and vortex.

9. Centrifuge for 10 min at 3500 rpm.

10. Transfer top layer to a clean test tube (to be dried down).

11. Add 2 mL of heptane to sample tube.

12. Vortex for 1 min followed by centrifugation for 10 min at 3500 rpm.

13. Transfer top layer to the clean tube (to be dried down).

14. Evaporate fatty acid methyl esters (FAME) + heptane (in clean tube) under nitrogen.

15. Resolubilize FAME in 200 µL helptane.

16. Transfer FAME to GC vial for storage.

[1]50 mg of tissue is preferred weight. Alternatively, up to 50 µL of aqueous sample (e.g. plasma, milk, red blood cells) may be used. Samples suspended in a greater volume of aqueous solution must first be dried using a Speed Vac concentrator.